CHARACTERIZATION AND COMPARISON OF VARIOUS IRON OXIDIZERS ISOLATED FROM INDIAN MINES FOR BIOHYDROMETALLURGICAL PROCESSES

THESIS SUBMITTED TO THE SAURASHTRA UNIVERSITY
FOR THE DEGREE OF

Doctor of Philosophy
in
Microbiology

BY

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RESEARCH GUIDE

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CERTIFICATE

This is to certify that the work presented in this thesis entitled **"Characterization and comparison of various iron oxidizers isolated from Indian mines for biohydrometallurgical processes"** submitted by **Parveen A. Ansari** has been carried out under my supervision in the Department of Microbiology, School of Sciences, Gujarat university, Ahmedabad, for the degree of Doctor of Philosophy in Microbiology.

I, further testify that this thesis or part thereof has not previously formed the basis of award of any degree, diploma, associateship, fellowship or any other similar awards.

Date :

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DECLARATION

Statement under O. Ph.D. 7 of Saurashtra University

I, ***Parveen A. Ansari***, the undersigned hereby solemnly declare that the work presented in the thesis entitled "**Characterization and comparison of various iron oxidizers isolated from indian mines for biohydrometallurgical processes"** is original and done by me. I declare further that this work has not been submitted for any degree or diploma to any other university or institution.

Date:

Place: **Parveen A. Ansari**

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Ansari Parveen Bano A.H.

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LIST OF ABBRIVIATIONS

$A_{260/280}$	Absorbance ratio of 260 nm and 280 nm
$A_{260/230}$	Absorbance ratio of 260 nm and 230 nm
ARDREA	Amplified ribosomal DNA restriction enzyme analysis
ATP	Adenosine triphosphate
AMD	Acid mine drainage
BDL	Below detection limit
bp	Base pairs
CFU	Colony forming unit
C	Centigrade
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxy ribonucleic acid
EDX	Energy dispersive X-ray analysis
FISH	Fluorescent *in situ* hybridization
g/L	Gram per litre
h	Hour
IOR	Iron oxidation rate
kb	Kilo basepair
K_m	Michaelis constant
K_s	Saturation constant
M	Molar
mg/L	Milligram per litre
ml	Milli litre
mM	Milli mole
mV	Milli volt
µ	Micron
ng	Nano gram
nm	Nano meter
ND	Not determined
O.D.	Optical density
ppm	Part per million
PCR	Polymerase chain reaction
RAPD	Random amplified polymorphic DNA
rDNA	Ribosomal deoxy ribonucleic acid
rpm	Rotation per minute
rRNA	Ribosomal ribonucleic acid
SEM	Scanning electron microscopy
t	Time
UPGMA	Unweighted pair group method of arithmetic means

UV	Ultra violet
V	Volt
V_{max}	Velocity maximum
%	Percentage

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Metals and minerals are undoubtedly the basic necessity in economic growth of a nation and no other substances have been as important as them in the story of man's control of his environment. The mining and refining of metals have formed a major aspect of production and social reproduction over the past six thousand years, and have employed millions of people throughout the old and new worlds (Craddock, 1995). All industrial societies are erected on foundations of metal, and still require vast quantities of metals (Lottermoser, 2007). It is generally agreed that the first known metals were gold, silver and copper (Encyclopaedia Britannica, 2009).

There is a continuous shortfall of high-grade economic minerals for metal extraction and this is more intensely felt in developing country proceeding toward industrialisation such as India. As against this, many developing nations are richly endowed with plenty of lean grade mineral reserves containing low amount of metals. When lean grade minerals are used for metal extraction, the traditional pyrometallurgy based metal recovery processes become economically less viable and new processes are required to be found out to work the remaining lower grade deposits.

In such situations, microbe based metal-extraction processes or "Biohydrometallurgy" have clear economic advantages in the extraction of metals from many low grade deposits. In this method extraction, concentration and recovery of metal is being done with help of microbes. The activity of microorganisms is regulated in a manner that it is beneficial to the mining industry and it can be exploited for commercial success.

The prokaryotic assemblage at mining environment is mainly composed of sulfur and iron oxidizing microorganisms. Microorganisms at these sites predominantly belong to the genus *Acidithiobacillus, Leptospirillum, Sulfobacillus, Sulfolobus, Acidianus* and *Acidiphilum.* Among these genera, mostly studied organisms are

extremely acidophilic, mesophilic, iron and sulfur oxidizers *Acidithiobacillus ferrooxidans, Acidithiobacillus thiooxidans and Leptospirillum ferrooxidans*.

Most prominent among the sulfide mineral leaching organism is *A. ferrooxidans*. This is a gram negative chemolitho-autotrophic bacterium that obtains energy from the oxidation of ferrous iron, elemental sulphur, reduced sulphur compounds such as elemental sulphur, tetrathionate, thiocynate and sulfide minerals. Many scientists have reported facultative autotrophic or mixotrophic nature of *A. ferrooxidans* strains. But afterwards they have been considered to occur due to presence of heterotrophic/mixotrophic contaminants (Harrison *et al.* 1980) and this organism is now placed as obligate autotroph in recent reclassification by Kelly and Wood (2000) and later on by Robertson and Kuenen (2006). Eventhough organic compounds are generally found to inhibit its growth, there have been many reports that many organic substances are beneficial to *A. ferrooxidans* and promote better growth.

A. ferrooxidans, typically live in environments that have high concentrations of soluble heavy metals (e.g., arsenic, mercury, and silver), as well as unusually high concentrations of potentially toxic metals (e.g., copper and iron). As a result *A. ferrooxidans* also has inherent resistance to high concentrations of metallic and other ions.

This organism is isolated in many locations worldwide where oxidizable iron, sulfide minerals and sulfur are exposed to atmospheric air. It is particularly prevalent in acid drainage waters of mines for sulfide mineral, mineral leach dumps and drainage waters from coal mines and coal or spoil heaps. Since their natural habitat are ecologically extremely diverse, different *Acidithiobacillus* strains of the same species developing in various ecological niches are characterized by differences in growth rate, tolerance to heavy metal ions and activities of ferrous iron and/or sulfide mineral oxidation.

The microbial diversity in mine samples is mostly evaluated by culture dependent methods. Direct bacterial counts and indirect measurements such as oxygen uptake in solid and liquid samples, redox potential, pH, ferrous iron concentration and temperature have provided useful information on the bulk activity of microorganisms.

Apart from conventional methods, culture independent approaches have produced significant advances in the field of microbial ecology. Molecular techniques such as mol% G+C content, DNA-DNA hybridization, PCR amplification, fluorescent in situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE) and 16S rRNA cloning and sequencing are used to analyze the microbial community inhabiting in mining environment.

The determination of the mol% G+C content of the DNA of bacterial isolates is also used to determine whether strains are related to each other. Same way the physiological behavior of an organism is found to be correlated with ubiquinone type. Efforts have been successful to differentiate 11 species of former genera *Thiobacillus* with the help of ubiquinones and DNA base compositions.

16S rRNA sequence in all organisms is considered to be conserved and it is used for measuring relatedness among species. DNA hybridization studies on *A. ferrooxidans*, *A. thiooxidans* and *A. thioparus* along with five bacteria formerly placed in *Thiobacillus* species established a high degree of homology (>70%) between strains of same species.

Based on the aforementioned facts, research work was undertaken to characterize a range of biomining bacteria isolated from various mines across India in order to ascertain their specific requirement and conditions suitable for its adaptation in biohydrometallurgical process.

2.1 INTRODUCTION

The present-day use of metals is the culmination of a long path of development extending over approximately 6,500 years. The origin of metallurgy goes back to a very early time, when people began melting certain rocks and minerals to obtain the new materials known as metals (Brombacher *et al.*, 1997).

The involvement of microorganisms in the mineral industry can be traced as far back as 1000 BC when the Romans, Phoenicians and other civilizations of the time leached permeable copper bearing rock with surface waters (Brombacher *et al.*, 1997). The copper sulfate containing surface water was then collected and copper was recovered by iron precipitation (Mwaba, 1993). However, the first example of *in situ* mining was documented around 166 AD by a Greek naturalist and physician - Galen concerning several copper mines of Cyprus. Even medieval writers such as Paracelsus, Valentine and Agricola, made reference to this leaching process in their works of the time (Brombacher *et al.*, 1997).

During the 16th century, metallurgical knowledge was recorded and made available. Two books were especially influential. One, by the Italian Vannoccio Biringuccio - a metal worker, was entitled *De la pirotechnia* (Eng. trans., *The Pirotechnia of Vannoccio Biringuccio*, 1943). This book dealt with smelting, refining, assay methods for determining the metal content of ores (Brombacher *et al.*, 1997). The other book entitled *De re metallica* was written by a German Georgius Agricola- a miner and an extractive metallurgist. This book described the processes used for crushing and concentrating the ore and also the methods of assaying to determine whether ores were worth mining and extracting (Encyclopaedia Britannica, 2009).

However, in the early 1920s, the oxidation of zinc sulfide by unidentified sulfur oxidizing microorganisms was reported, suggesting a biological method for an economical utilization of low grade zinc

sulfide ores (Brombacher *et al.*, 1997). Colmer and Hinkle in 1947 isolated and described bacteria, which could enormously accelerate the oxidation of ferrous iron. The earliest comprehensive account of the fundamental biogeochemical phenomena was given by Silverman and Ehrlich in 1964. To make best use of these mineral oxidizing microbes in mineral recovery, extensive knowledge of mineral reservoirs above and below the surface of earth is required.

2.2 PHASES OF EARTH

The surface of the earth can be divided into 3 distinct phases:

1. Lithosphere - the crust of the earth,
2. Hydrosphere - the aqueous portion of the earth's surface including the oceans, seas, lakes and rivers,
3. Atmosphere - the gaseous envelop around the globe.

Portions or the whole of each of these phases are included in the biosphere - the portion of the earth's surface, which is inhabited by living organisms. Ninety eight percent of the crust is dominated by O, Si, Al, Fe, Mg, Na and K. These elements occur primarily in rocks and sediments (Ehrlich, 1981). There are 2 major sources of minerals:
(A) The terrestrial or land deposits and (B) Marine deposits.

The land deposits are getting exhausted due to continuous mining activities and even though vast tracts of unexplored regions still exist all over the world due to one reason or another, their exploitation is far from being achieved in the near future and a continuous short-fall in increasing term is being felt for all economic minerals and this is more intense in a developing country proceeding toward industrialization such as India.

The marine deposits are being worked out recently in the form of manganese nodules (Kumari and Natarajan, 2001; Kumari and Natarajan, 2002 (a) and 2002 (b)). These are relatively inexhaustible as they accumulate in oceans at rate greater than their present rate of

consumption. But the technology is still in a development process. Hence to excavate and obtain maximum minerals from available sources, technology development of mining operations is required.

2.3 MINING OPERATIONS

Operation in mining industry include:

1. **Mining** is the first operation in the commercial exploitation of a mineral or energy resource. It is defined as "the extraction of material from the ground in order to recover one or more component parts of the mined material".

2. **Mineral processing** or beneficiation aims to physically separate and concentrate the ore mineral(s). Metal ores consist of coherent mixtures of minerals of value and other gangue material. To recover and process the target minerals, processing is done. This includes activities like grinding, separation by gravity, magnetic or electrostatic method and floatation (Natarajan and Deo, 2001; Okibe and Johnson, 2002; Natarajan and Das, 2003; Patra and Natarajan, 2004; Chandraprabha *et al.*, 2005).

3. **Metal extraction** is done by destroying the crystallographic bonds in the ore mineral in order to recover the desired element or compound (Lottermoser, 2007).

2.3.1 Conventional extraction method: Pyrometallurgy

Traditionally, metals are extracted by 'smelting' or pyrometallurgy, the thermal treatment of minerals and metallurgical ores and concentrates to bring about physical and chemical transformations, which then enables recovery of valuable metals (Siezen and Wilson, 2009). These methods involve roasting, smelting, pressure cyanidation, pressure acid leaching and digestion in nitric acid / mixed acids which liberate precious (e.g. gold) and base (e.g. copper) metals trapped in metal sulfides.

However, conventional pyrometallurgical processes like roasting and smelting emit sulfur dioxide and volatile heavy metals e.g lead and arsenic in the environment. Smelting operations are estimated to produce 8% of the worldwide sulfur emissions (Mackenzie and Masten, 2004). In pressure oxidation and cyanidation, very high temperature and pressure are required, which are unfavorable due to energy cost and enhanced corrosion.

2.3.2 Biohydrometallurgy

As high grade surface mineral deposits are worked out, the traditional pyrometallurgy based metal recovery processes become economically less viable and new processes are required to be found out to work the remaining lower grade deposits. Microbe based metal-extraction processes or **Biohydrometallurgy** have clear economic advantages in the extraction of metals from many low grade deposits (Rawlings *et al.*, 2003).

Biohydrometallurgy is an interdisciplinary subject combining geomicrobiology, microbial ecology, microbial biogeochemistry and hydrometallurgy (Brombacher *et al.*, 1997). This technology has been proved to be cheaper, more efficient and environmentally friendly than roasting and high pressure moisture heating processes. This technique does not use the large amounts of energy required by roasting or smelting. It also does not produce sulfur dioxide and other harmful gaseous emissions (Makita *et al.*, 2004).

In biohydrometallurgical method - extraction, concentration and recovery of metal is being done with help of microbes. In the ore, medium of water allows the microorganisms to grow in and around ore particles and carry out conversion, dissolution and precipitation of metal compounds and metals. This technology is especially helpful for developing nations, where being less expensive and less sophisticated in handling as well as technology is desirable for its translation into field scale practice.

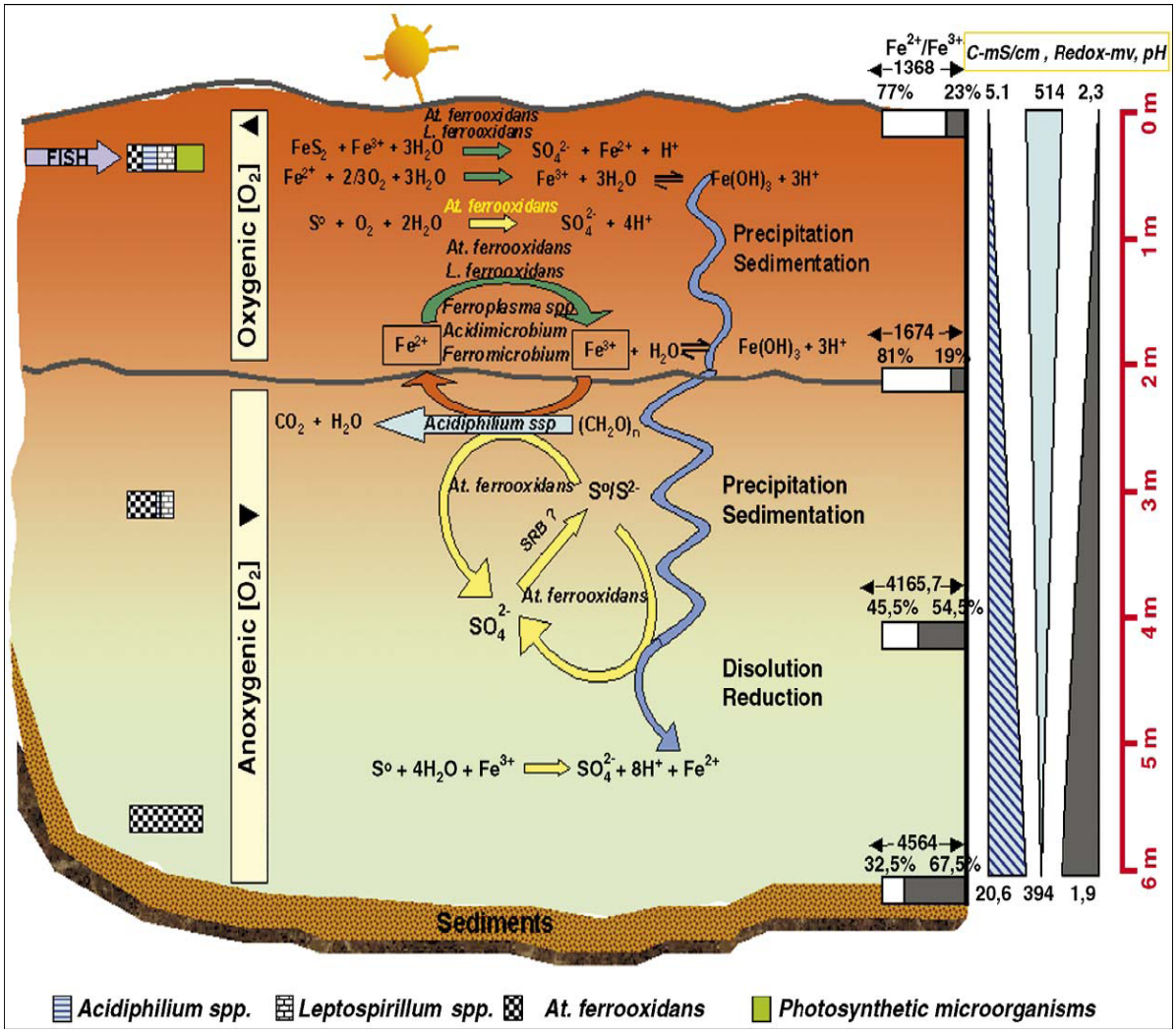
2.4 BIOMINING MICROORGANISMS

The complex microbial populations associated with leaching environments are dominated by microorganisms, which apart from sulfur and iron oxidizing capabilities, tolerate high acidity levels and high metal ion concentrations (Mwaba, 1993). These iron-oxidizing bacteria are useful in biotechnologies of heavy metal removal from shale ore, polluted soils and sewage sludge containing organic hazardous compounds (Matlakowska and Sklodowska, 2007). As many as 86 strains falling in various genera have been identified from acidic environments (pH <4.5) by Robbins 2000.

Microorganisms at these sites predominantly belong to the genus *Acidithiobacillus, Leptospirillum, Sulfobacillus, Sulfolobus, Acidianus* and *Acidiphilum* (Ralph, 1982; Karavaiko *et al.*, 1988; Dopson and Lindstrom, 1999; Rawlings and Johnson, 2007). Their interrelated metabolic activity in environment, as detected by DGGE, FISH and cloning are shown in figure 2.1 (Malki *et al.*, 2006). *Acidithiobacillus ferrooxidans* is considered one of the most important microorganisms in the bioleaching of sulfide ores. This might be due to its ability to utilize both Fe^{2+} and sulfur moieties in sulfide ores for growth (Sugio *et al.*, 2008). Table 1.1 enlists physiological properties of metal sulfide oxidizing, acidophilic microorganisms (Johnson *et al.*, 2005; Schippers, 2007). The contribution of microorganisms like yeast, fungi and algae associated with the minerals has also been demonstrated by many investigators (Dave *et al.*, 1981; Dave and Natarajan, 1987).

The microorganisms implicated in sulfide mineral dissolution have different physiological requirements. For example, *A. ferrooxidans* is iron & sulfur oxidizing aerobic organism, which grows best at pH 1.5 – 3 at mesophilic environment 25°C to 30°C (Gomez *et al.*, 1999), while *L. ferrooxidans* cannot oxidize sulfur and it grows at slightly higher

Fig. 2.1: Model system of the different chemolithotrophic activities relevant to biohydrometallurgy processes. (Malki *et al.*, 2006)



temperatures i.e. 30°C - 45°C. At initial stages of leaching, ferric to ferrous iron concentration is low and hence *A. ferrooxidans* will be dominant. At later stages of bioleaching, ferric iron will accumulate and *Letospirillum* will outgrow *Thiobacilli* (Rawlings *et al.*, 1999; Dave, 2008). While *A. thiooxidans* grows best at 25°C to 30°C temperature and pH 1.5 – 3, does not oxidize iron and derives its energy by oxidation of reduced sulfur compounds.

2.4.1 Characteristics of the earlier genus *Thiobacillus* Beijerinck (1904)

Majority of biomining organisms were included in a common genus *Thiobacillus* Beijerinck till the year 2000. The species of genus *Thiobacillus* fell into the 'α-', 'β-' and 'γ-' subclasses of the *Proteobacteria* and the type species *Thiobacillus thioparus* was located in β-subclass.

All were small, gram negative rod-shaped bacteria (0.3-0.5 X 0.7-4.0 μm), some species being motile by means of polar flagella. All species could fix carbon-dioxide by means of Calvin-Benson cycle.

The species included in '*Thiobacillus*' genus exhibited almost as much diversity in DNA composition and physiology as was found collectively in all other proteobacterial groups e.g. tolerance of pH from 0 - > 8.5 (with pH and temperature optima of <2-8 and 20-50 °C, respectively), diverse G+C content of the DNA (50-68 mol%), diversity of DNA homology and range of ubiquinones and fatty acids (Kelly and Wood, 2000). This diversity of properties among species was indicative of an extremely heterogenous group.

2.4.2 Revised characterization of the genus *Acidithiobacillus*

On the basis of physiological characters and 16S rRNA gene sequence comparisons several members of *Thiobacillus* have been transferred to other genera while eight species were grouped in three new designated genera within the γ-subclass of the Proteobacteria, namely *Acidithiobacillus, Halothiobacillus* and *Thermithiobacillus* as shown in table 2.2 (Kelly and Wood, 2000; Kelly *et al.*, 2007).

The genus *Acidithiobacillus* was established by Kelly and Wood (2000), with *A. thiooxidans* (formerly *Thiobacillus thiooxidans*) as the type species. The 4 species included in the genus are gram-negative, rod shaped (0.4-2μm), motile with one or 2 flagella. Key features of the type strains of all the 8 species belonging to genera *Acidithiobacillus, Halothiobacillus and Thermithiobacillus* is given in table 2.3 (Schippers, 2007).

Table 2.2 : Reclassification and renaming of species of earlier genera *Thiobacillus*. (Kelly and Wood, 2000; Kelly *et al.*, 2007)

New genera	***Revised nomenclature***	***Old nomenclature***
Acidi-thiobacillus	*Acidithiobacillus thiooxidans*	*Thiobacillus thiooxidans*
	Acidithiobacillus ferrooxidans	*Thiobacillus ferrooxidans*
	Acidithiobacillus caldus	*Thiobacillus caldus*
	Acidithiobacillus albertensis	*Thiobacillus albertensis*
Halo-thiobacillus	*Halothiobacillus neopolitanus*	*Thiobacillus neopolitanus*
	Halothiobacillus halophilus	*Thiobacillus halophilus*
	Halothiobacillus hydrothermalis	*Thiobacillus hydrothermalis*
Thermi-thiobacillus	*Thermithiobacillus tepidarius*	*Thiobacillus tepidarius*
Thiomonas	*Thiomonas intermedius*	*Thiobacillus intermedius*
	Thiomonas perometabolis	*Thiobacillus perometabolis*
	Thiomonas thermosulfatus	*Thiobacillus thermosulfatus*
	Thiomonas cuprinus	*Thiobacillus cuprinus*
(Genera modified)	*Paracoccus versutus*	*Thiobacillus versutus*
	Acidiphilium acidophilum	*Thiobacillus acidophilus*
(Genera retained)	*Thiobacillus thioparus*	*Thiobacillus thioparus*
	Thiobacillus denitrificans	*Thiobacillus denitrificans*
	Thiobacillus aquaesulis	*Thiobacillus aquaesulis*
	Thiobacillus plumbophilus	*Thiobacillus plumbophilus*
	Thiobaillus novellus	*Thiobaillus novellus*
	Thiobacillus prosperous	*Thiobacillus prosperous*
	Thiobacillus delicatus	*Thiobacillus delicates*

2.4.3 Characteristics used in classification of Acidit*hiobacillus*

1. **Phenotypic characters:** Many of the phenotypic characters of *Acidithiobacilli* such as rod shape, motility, Gram-negative reaction and utilization of sulphur compounds are common with species formerly placed in *Thiobacillus*. These characters are useful for broad recognition but not for critical identification.

2. **Mol % G+C content:** The mol% G+C determination can relate the strains to each other. G+C values for the different species of *Acidithiobacillus* are often sufficiently far apart to serve as useful species characteristics. Table 2.4 enlists mol% G+C value of metal sulfide oxidizing, acidophilic microorganisms.

3. **Ubiquinones and cellular fatty acid analysis:** The physiological behaviour is found to be correlated with ubiquinone type. 11 species of former genera *Thiobacillus* could be differentiated with the help of ubiquinones and DNA base compositions. All the species presently assigned to *Acidithiobacillus* possess 8 isoprene units but 1 strain of *A. ferrooxidans* is reported to have 8 as well as 9 isoprene units (Johnson *et al.*, 2005).

4. **Nucleotide structure:** 16S rRNA sequences are considered to be conserved and are used for measuring relatedness among species. Microbes with 16S rRNA sequence similarity upto 97% identical are considered as members of the same genus. Similarities within the sequences have enabled the species of previous genus *Thiobacillus* to be assigned to α, β or γ groups of proteobacteria. As shown in figure 2.2, microbial community comprising microbes from 11 putative divisions have been detected by sequencing 16S ribosomal RNA genes (16S rRNA) from environmental samples (Baker and Banfield, 2003).

5. **DNA homologies:** DNA hybridization studies on *A. ferrooxidans*, *A. thiooxidans* and *A. thioparus* with five bacteria formerly placed in *Thiobacillus* species established a high degree of homology (>70%) between strains of same species.

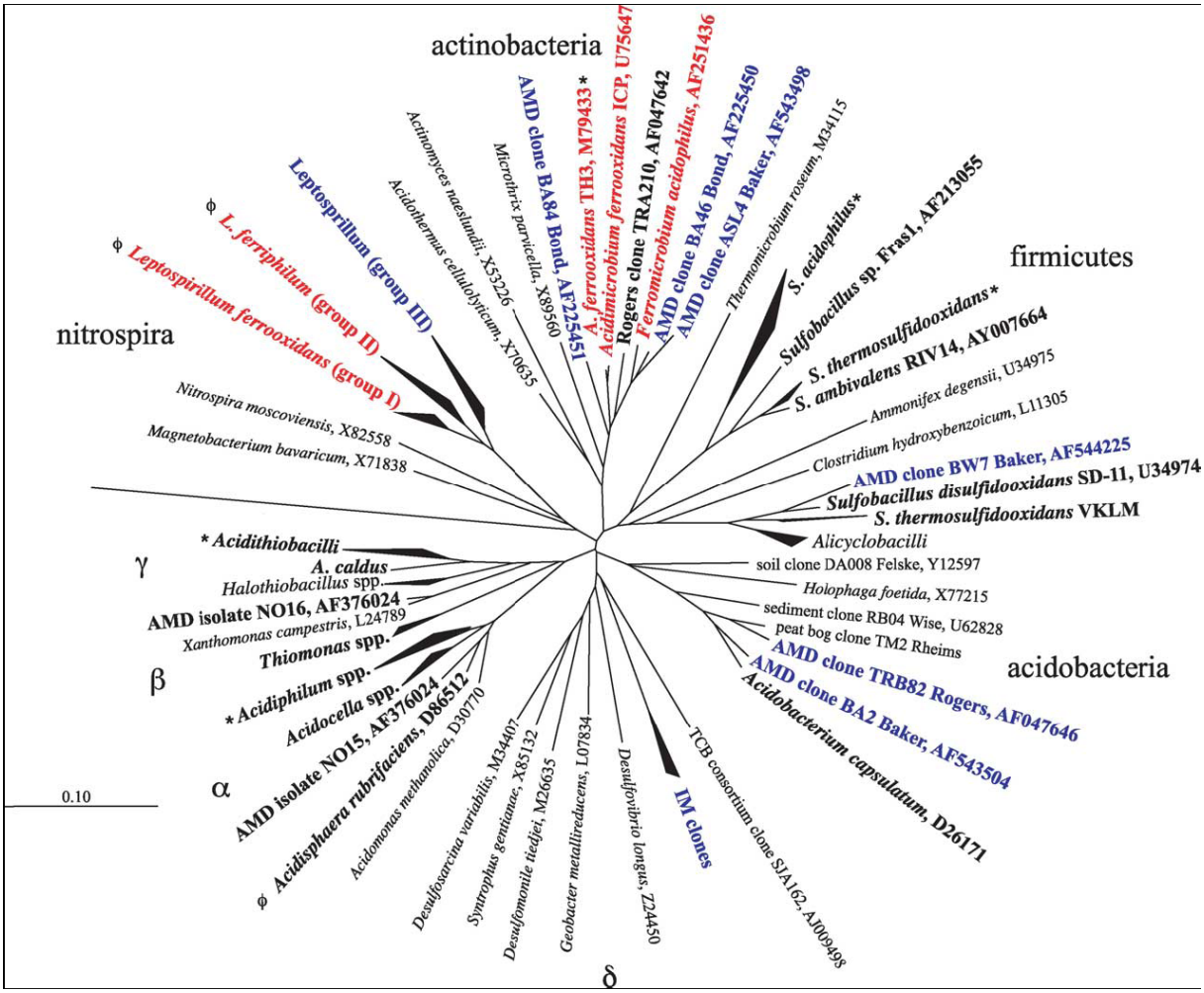
Table 2.4 : Phylogeny of metal sulfide oxidizing, acidophilic micro-organisms. (Johnson *et al.*, 2005; Schippers, 2007)

Species#	Phylum	G+C (mol%)
Mesophilic and moderately thermophilic bacteria		
Acidimicrobium ferrooxidans	Actinobacteria	67-69
Acidithiobacillus albertensis	Proteobacteria	61.5
Acidithiobacillus caldus	Proteobacteria	63-64
Acidithiobacillus ferrooxidans	Proteobacteria	58-59
Acidithiobacillus thiooxidans	Proteobacteria	52
Alicyclobacillus disulfidooxidans	Firmicutes	53
Alicyclobacillus tolerans	Firmicutes	49
"Caldibacillus ferrivorus"	Firmicutes	51
"Ferrimicrobium acidiphilum"	Actinobacteria	51-55
Leptospirillum ferriphilum	Nitrospira	55-58
"Leptospirillum ferrodiazotrophum"	Nitrospira	Na
Leptospirillum ferrooxidans	Nitrospira	52
Sulfobacillus acidophilus	Firmicutes	55-57
"Sulfobacillus montserratensis"	Firmicutes	52
Sulfobacillus sibiricus	Firmicutes	48
Sulfobacillus thermosulfidooxidans	Firmicutes	48-50
Sulfobacillus thermotolerans	Firmicutes	48
"Thiobacillus plumbophilus"	Proteobacteria	66
"Thiobacillus prosperus"	Proteobacteria	64
Thiomonas cuprina	Proteobacteria	66-69
Mesophilic and moderately thermophilic archaea		
"Ferroplasma acidarmanus"	Euryarchaeota	37
Ferroplasma acidiphilum	Euryarchaeota	36.5
"Ferroplasma cupricumulans"	Euryarchaeota	Na
Extremely thermophilic archaea		
Acidianus brierleyi	Crenarchaeota	31
Acidianus infernus	Crenarchaeota	31
Metallosphaera hakonensis	Crenarchaeota	46
Metallosphaera prunae	Crenarchaeota	46
Metallosphaera sedula	Crenarchaeota	45
Sulfolobus metallicus	Crenarchaeota	38
Sulfolobus yangmingensis	Crenarchaeota	42
Sulfurococcus mirabilis	Crenarchaeota	~44
Sulfurococcus yellowstonensis	Crenarchaeota	45

Listed in alphabetical order

Species without standing in nomenclature are given in quotation marks

Fig. 2.2 : Phylogeny of prokaryotic 16S rRNA genes from acid mine drainage and bioleaching sites (in bold) with reference lineages. (Baker and Banfield, 2003)



* indicate lineages known to contain facultative anaerobes

ϕ appear to be obligate aerobes.

The lineages in blue have no cultivated members to date.

Lineages in red do not utilize sulfur, only iron oxidizers.

Putative divisions are shown near each of their branchings, the β-, γ-, δ-, α-proteobacteria are shown.

2.4.4 Molecular biology of the *Acidithiobacilli*

Acidithiobacillus ferrooxidans type strain ATCC23270 is the first biomining microorganism to have had its genome almost completely sequenced. Irazabal *et al.* (1997) constructed the first complete and highly resolved physical map (86 restriction sites) of *A. ferrooxidans* chromosome, with more than 2700 identified open reading frames (ORFs) in about 3 million base pairs (bp) of genome size (Barretto *et al.*, 2003). The sequence and annotation of the complete *A. ferrooxidans* strain ATCC 23270 genome is available at the Comprehensive Microbial Resource (CMR) (J. Craig Venter Institute, http://www.jcvi.org) and in GenBank/ EMBL/DDBJ accession number CP001219 (Valdes 2008). An analysis of the genome of the type strain is shown in table 2.5 and it provides a coherent view of its gene content and metabolic potential.

Bioinformatics analysis of the genome provides a valuable platform for gene discovery i.e. genome-wide search "*in silico*" for candidate genes and functional predictions that helps explain the activity of *A. ferrooxidans* in industrial bioleaching. This information can then be used to reconstruct metabolic pathways and to understand the often complicated and multilevel regulation of cellular functions. Genes involved in phosphate, sulfur and iron metabolism, quorum sensing, metal resistance, amino acid biosynthesis pathways and formation of EPS precursors have been studied (Zeng *et al.*, 2007).

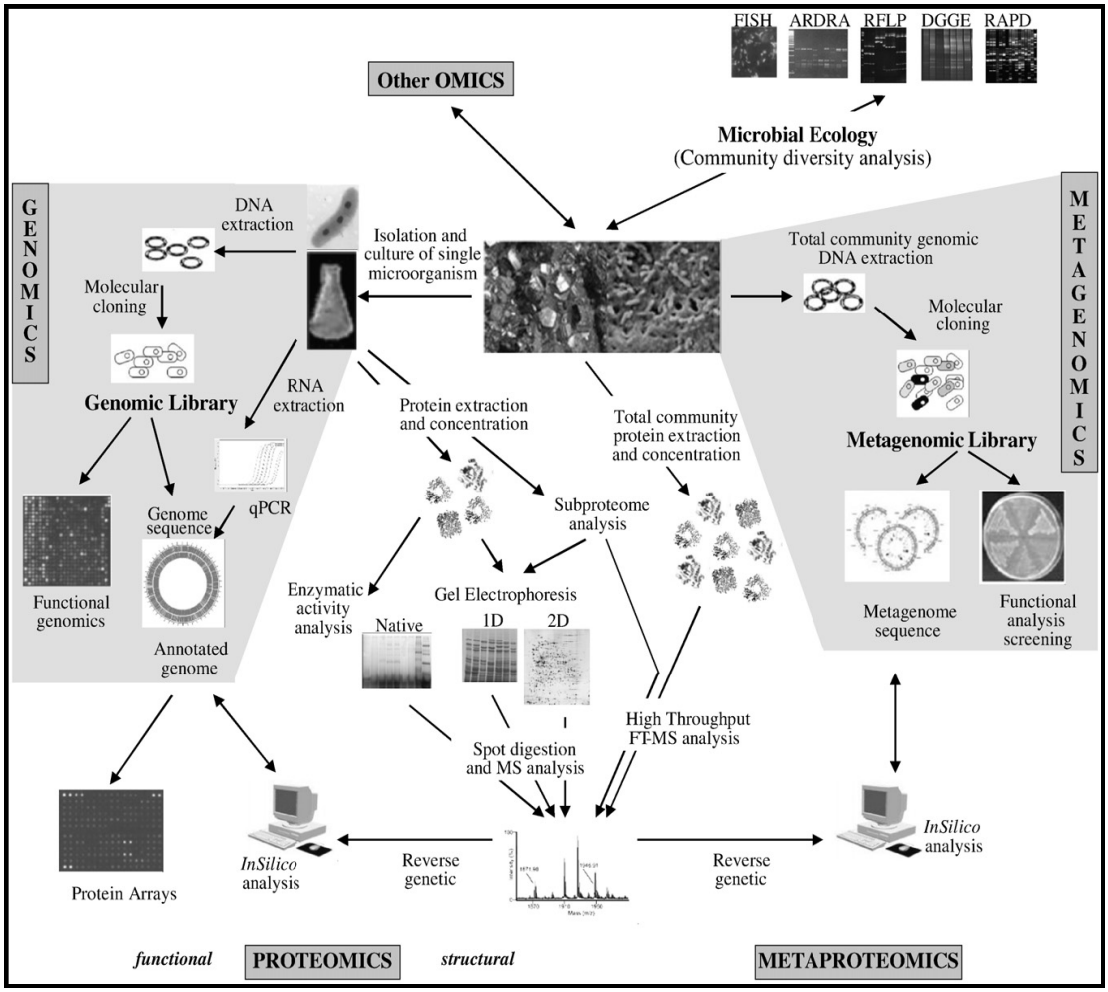
However all these bioinformatic predictions will have to be demonstrated experimentally by using functional genomics, proteomics and other approaches. A schematic overview of various molecular biology tools to study biomining community is depicted in figure 2.3. **Functional genomics** is a culture dependent method, which involves the experimental confirmation of the activity carried out by the expression of the genes found out by bioinformatics. While **Metagenomics** is culture independent method, and involves whole genomic analysis of a microbial community.

Table 2.5: General features of the *A. ferrooxidans* ATCC23270 genome. (Valdes 2008)

Characteristic	**Value**
Complete genome size, bp	2,982,397
G+C percent (%)	58.77
Total No. of coding proteins	3,217
Coding density (%)	97.45
No. of rRNA operons (16S-23S-5S)	2
No. of tRNA genes	78
Proteins with known functions	2070
Conserved hypothetical proteins	388
Hypothetical proteins	759
Most represented functional categories	**(%)**
Cell envelope	7.8
Transport and binding proteins	7.61
Energy metabolism	6.52
Best BLASTP comparisons against complete proteomes	**Number of best blast hits**
γ-proteobacteria	899
β-proteobacteria	791
α-proteobacteria	271
δ-proteobacteria	103
Cyanobacteria	73
Archaea	41

The development of culture independent technologies such as (PCR) fingerprinting, reverse transcriptase PCR, real time PCR, reporter genes and fluorescent in situ hybridization (FISH) have made it possible to study the diversity in natural settings (Schrenk *et al.*, 1998; Engel *et al.*, 2001; Jackson *et al.*, 2001; Gentry *et al.*, 2006; Hirayama *et al.*, 2007, Rusch and Amend, 2007; Ni *et al.*, 2008; Ferraz *et al.*, 2010).

Fig. 2.3 : A schematic overview of various molecular biology tools to study biomining community. (Valdes 2008)



2.4.5 Genetic manipulation in *A. ferrooxidans*

The molecular research on *Acidithiobacilli* has proved to be a considerable technical challenge because of its acidophilic chemolithotrophic nature, lack of distinguishable molecular markers, recalcitrance to cellular lysis and low cell yield in microbiological culture media (Chisholm *et al.* 1998; Mackenzie and Masten, 2004).

Strain improvement of *Acidithiobacilli* by recombinant DNA technology is being pursued to improve substrate specificity, metal ion resistance and growth rates. Many strains of *A. ferrooxidans* harbor plasmid DNA. Because of the fact that in many bacteria the resistance of

metals is mediated by plasmids, it has been suggested that plasmids are also involved in resistance to metals in *A. ferrooxidans* (Chisholm *et al.*, 1998). Dominy *et al.*, (1997) have isolated and cloned a 19.8 kb plasmid- pTF5 in *E. coli* having genes for electron transfer proteins. Kondrat'eva *et al.* (2004) carried out the restriction analysis of two plasmids pTFK1 and pTFK2 of the *A. ferrooxidans* and found out the sizes of these plasmid to be 1.5 and 30 kb.

Most plasmid borne selective markers (e.g. antibiotics) are not effective under the culturing conditions of high acid, metal or sulfur compound concentration required by *A. ferrooxidans.* No mutant strains are available for complementation markers and mutant selection in *A. ferrooxidans* is limited because of its autotrophic nature. Testing of putative cloning vectors, therefore, has been hampered by the lack of useful selection markers, mutants and effective gene-transfer techniques (Mackenzie and Masten, 2004).

2.4.6 Diversity of iron –oxidizing microbes

Some of the diverse iron-oxidizing bacteria have yet to be placed in the context of biomining and could be active in mineral processing operations under conditions not generally used in the laboratory with the more familiar species (Norris, 2007). These conditions include higher temperature, extremes of pH, the presence of organic substrates and salinity.

2.4.6.1 High temperature

The potential application of thermophilic, *acidithiobacillus*-like bacteria (TH – 1, 2, 3) have been the subject of renewed interest since many years (Brierley *et al.*, 1978; Karavaiko, 1988, Cleaver *et al.*, 2007). Among them TH-1 was classified as *Sulfobacillus thermosulfidooxidans a*nd TH-3 was designated as *Acidimicrobium ferrooxidans* (Clark and Norris, 1995; Dopson and Lindstrom, 1999; Clum *et al.*, 2009). Of great interest are moderate to extreme thermophilic microorganisms isolated from hot springs like *A. caldus,*

Acidianus brierleyi, Sulfolobus acidocaldarius and species of *Metallosphaera* and *Sulfurisphaera* which are capable of growth at temperatures in the range 45°C - 80°C and pH ranging from 1 to 6 (Brierley *et al.*, 1978; Konishi *et al.*, 1995). Ferroni *et al.* (1986) had isolated psychrotrophic strain of *A. ferrooxidans* from uranium mines.

2.4.6.2 Extremes of acidity

Strains and/or species of archaea *Ferroplasma* are favored by low pH and organic carbon source (Hawkes *et al.*, 2006). Their widespread distribution is observed in natural geothermal environments, industrial mineral processing bioreactors and ore leaching heaps. Mesophilic *Ferroplasma acidiphilum,* originally isolated from pyrite/arsenopyrite processing pilot plant, grows optimally at pH 1.7, whereas *Ferroplasma acidarmanus* grows below pH 1 (Pivovarova *et al.*, 2002; Olson *et al.*, 2003; Okibe *et al.*, 2003; Hawkes, 2006).

Moderate acidophilic, iron oxidizing bacteria have been found in mine drainage above pH 3 including species that cluster phylogenetically with *Thiomonas* and *Halothiobacillus* species and some of them are known to oxidize As^{3+}(Moreira and Amils, 1997; Emerson and Moyer, 1997; Battaglia *et al.*, 2002; Bruneel, 2003; Chen *et al.*, 2004; Popa and Kinkle, 2004; Bruneel *et al.*, 2005; Duquesne, 2008). At higher temperature, the bacterium *Hydrogenobaculum acidophilum* (previously named *Hydrogenobacter acidophilus*) is found, which has an optimum pH of 3-4.

2.4.6.3 Presence of organic substrate

Obligately heterotrophic, iron-oxidizing acidophiles have been visible mainly in acid mine drainage environments (Johnson *et al.*, 1992; Nicolau and Johnson, 1999). The mesophile *Ferrimicrobium acidiphilum* is Gram positive and more related to *Acidimicrobium ferrooxidans* rather than to gram negative, proteobacterial iron-oxidizing mesophiles. Other Gram positive, heterotrophic, iron-oxidizing acidophiles are related to the *Sulfobacillus* genus but more

closely to the *Alicyclobacillus* (Inoue *et al.*, 2002). Facultatively organotrophic and acidophilic, metal mobilizing *Thiobacillus cuprinus* was reported by Huber and Stetter (1990).

2.4.6.4 Salt tolerant species

Most acidophiles are sensitive to salt (NaCl), but several species of halotolerant, iron-oxidizing acidophiles isolated from marine geothermal sites are found in the *Thiobacillus prosperus* group (Huber and Stetter, 1989). *Thiobacillus prosperous* was reported as the first marine metal mobilizing bacterium (De Siloniz *et al.*, 1993).Other halotolerant bacteria similar to *T. prosperus* strains and salt tolerant, iron-oxidizing *Alicyclobacillus*-like bacteria have been isolated from marine harbor sediments. A new species- *Halothiobacillus kellyi* was reported by Sievert *et al.* (2000) from hydrothermal vent, which was obligately chemolithotroph.

2.5 BACTERIAL OXIDATION OF IRON

Iron is the fourth most abundant element in the earth's crust, and is the most abundant element in the earth as a whole. Its average concentration in the crust is 5%. It is found in a number of minerals in rocks, soils and sediments. Iron is a very reactive element. It can exist in oxidation states of 0, +2 and +3. At pH values greater than 5.0, its ferrous form (+2) is readily oxidized in air to the ferric form (+3). Under reducing conditions, ferric iron is readily reduced to the ferrous state.

In acid solutions, metallic iron readily oxidizes to ferrous iron with the production of hydrogen.

$Fe^{\circ} + 2H^{+} \rightarrow Fe^{2+} + H_2$.....(1)

Ferric iron precipitates in alkaline solution and dissolves in acid solution.

It has been established that for a number of microorganisms, iron serves as the source of energy. Microbially catalyzed oxidation of iron can be represented by the reaction (Lundgren and Malouf, 1983; Mwaba, 1993; Rawlings and Silver, 1995):

$$4\ FeSO_4 + O_2 + 2\ H_2SO_4 \xrightarrow{\text{bacteria}} 2\ Fe_2(SO_4)_3 + 2H_2O \quad \ldots\ldots\ldots\ldots\ldots(2)$$

It is now widely accepted that the above reaction accurately describes the overall process of iron oxidation by acidophilic bacteria. Since Fe^{2+} as the growth substrate furnishes only a limited amount of energy, large quantities of ferrous iron are required for the process: approximately 2 X 10^6 atoms of Fe^{2+} are oxidized per cell per second (Karavaiko, 1988).

2.5.1 MICROBIAL IRON TRANSFORMATION IN NATURE: THE IRON CYCLE

Microbial transformation of iron plays an important role in cycling of iron in nature (Fig. 2.4). Iron is introduced into the cycle through the bacterial and chemical weathering of iron containing minerals in rocks, soils and sediments. The liberated iron, if ferrous, may be biologically or non-biologically oxidized to ferric iron under partially or fully aerobic conditions. The oxidation may be immediately followed by precipitation of the iron as a hydroxide, oxide, phosphate or sulfate (Lundgren and Malouf, 1983).

If complexing agents such as humic substances are present, the ferric iron may be converted to soluble complexes and is dispersed from its site of formation. In hot, humid climates, ferric iron is precipitated at the site of its release from soil mineral. This is due to the intense microbial action which rapidly and completely oxidizes available organic matter and prevents the formation of soluble organic ferric complexes. The iron precipitates thus formed tend to cement soil particles together in a process known as **"laterization"** (Lundgren and Malouf, 1983).

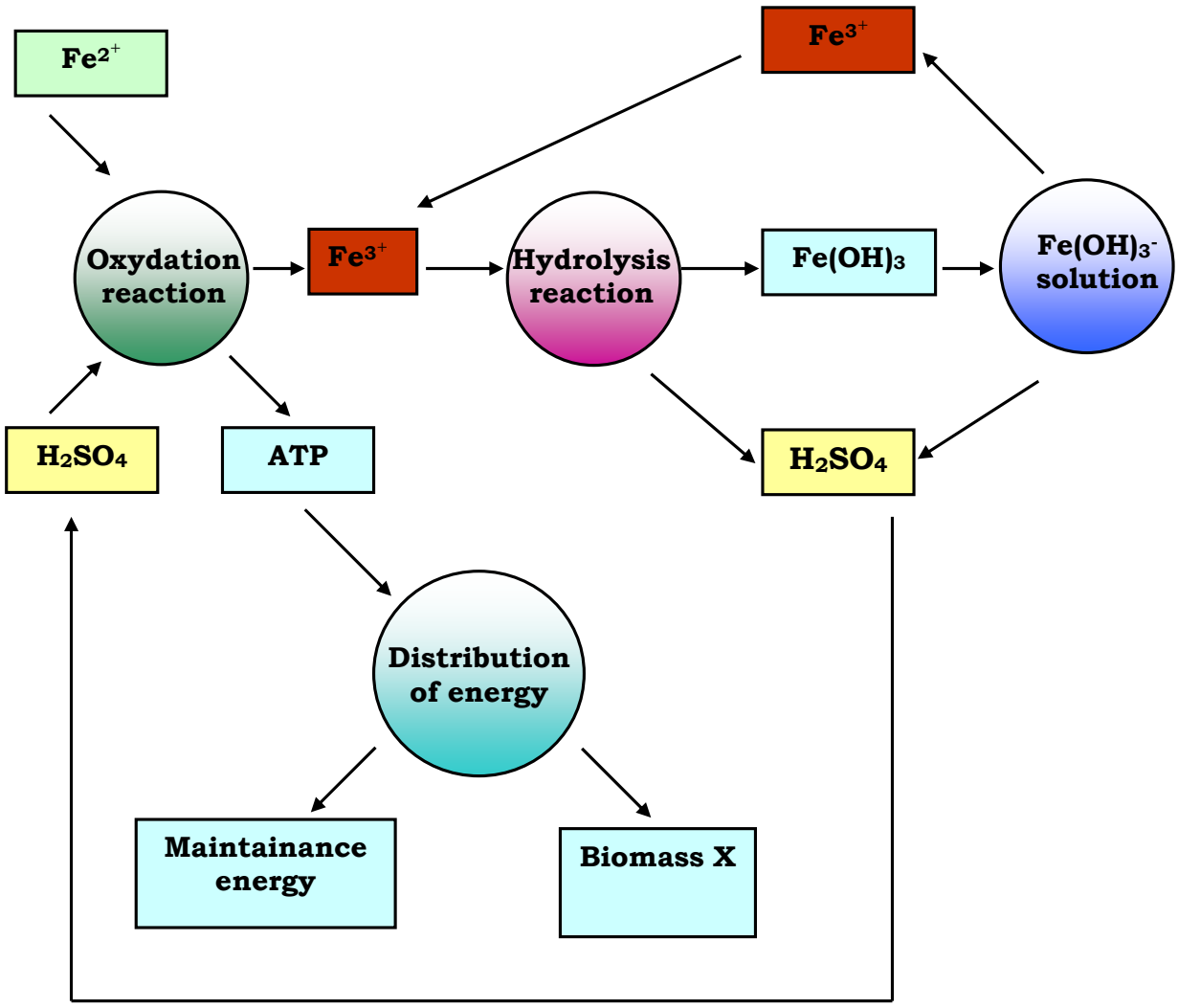
2.5.2 IRON OXIDISING ENZYME SYSTEMS

The search of the enzymatic components involved in Fe^{2+} oxidation has been undertaken by a number of investigators (Carlos 2008). All bacteria have multiple branched respiratory pathways with a diverse range of electron donors and acceptors (Fig 2.5). Among these electron transporters, cytochromes c are widely distributed in eukarya, archaea and eubacteria. The terminal electron acceptor is assumed to be a cytochrome oxidase anchored to the cytoplasmic membrane. *A. ferrooxidans* exhibits an unusually high content of cytochromes c, which can constitute as much as 10% of the total proteins synthesized in cells grown on Fe^{2+} (Yarzabal, 2002).

An early study of the Fe^{2+} oxidizing enzyme system in cell free extracts of a strain of *A. ferrooxidans* revealed the presence of cytochrome c and cytochrome a_1 and either traces or no cytochrome b (Blake *et al.*, 1992). The enzymes and protein involved in iron oxidation is given in figure 2.5 (Valdes 2008). Ferrous iron is oxidized in the periplasmic space of the cell envelop by a copper protein-rusticyanin, which transfers its electrons to cytochrome c. This cytochrome shuttles between the periplasmic space and the cell membrane, where it passes its electrons to cytochrome a, on the inner surface of the plasma membrane. At the same time, the electrons are passed inward through the membrane protons and oxygen pass in the same direction to be combined into water by cytochrome a_1. Hence, *A. ferrooxidans* respiratory chain follows the pathway: Fe(II) $\rightarrow$ Cyc2 $\rightarrow$ rusticyanin $\rightarrow$ cytochrome c4 $\rightarrow$ cytochrome oxidase aa3 $\rightarrow$ O_2 (Carlos 2008).

These proteins are encoded by the rus operon genes, which are arranged in the *A. ferrooxidans* genome in the following way: cyc2, cyc1, ORF1, coxB, coxA, coxC, coxD, and rus. The cyc2 gene encodes a c-type cytochrome localized in the outer membrane, the cyc1 gene encodes a periplasmic cytochrome c4 (or cytochrome c552), and ORF1 apparently encodes a putative outer membrane protein with unknown function.

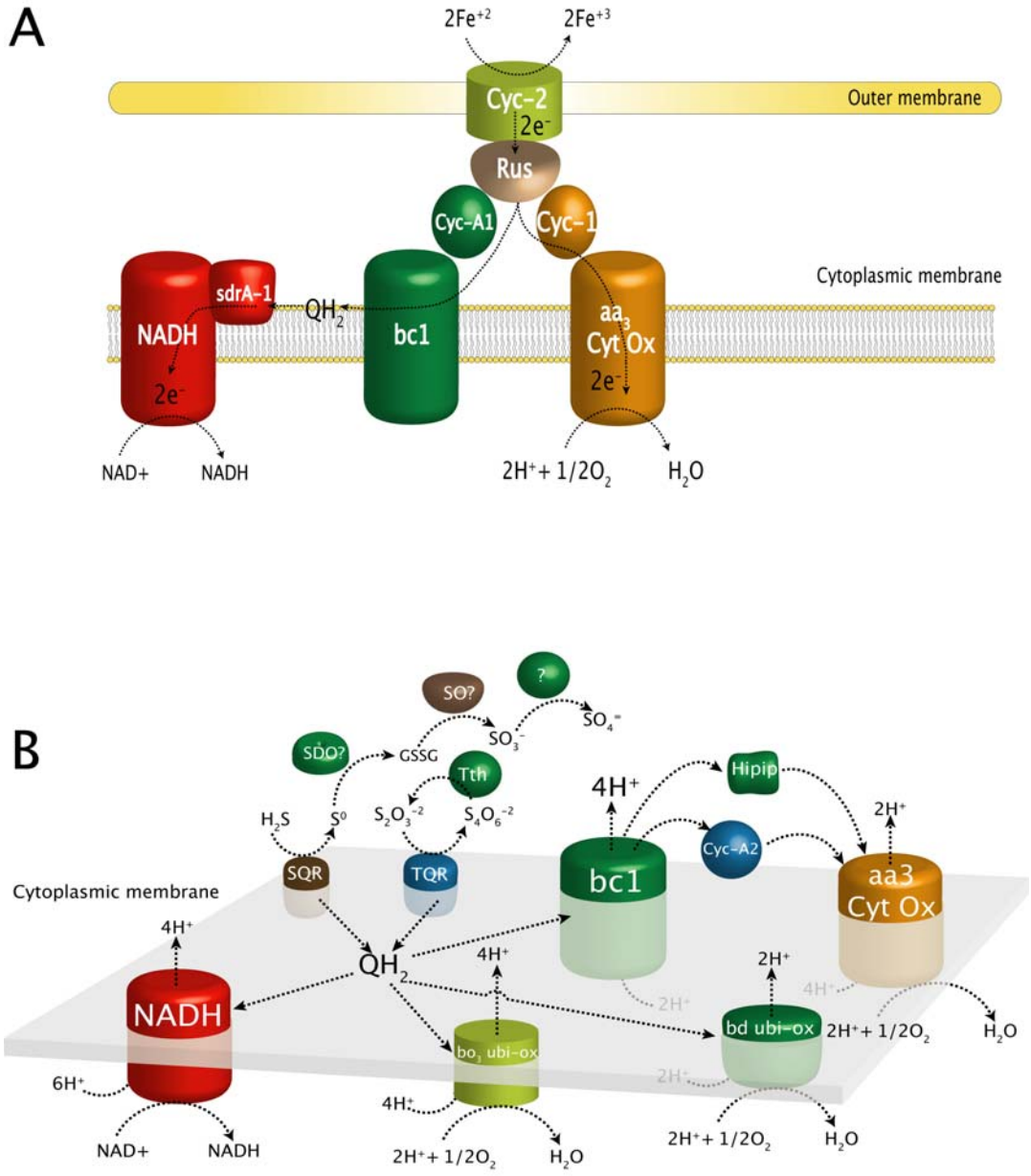
Fig 2.4 : Block diagram of *A. ferrooxidans* growth and Fe^{2+} oxidation. (Lundgren and Malouf, 1983)



The coxBACD genes encode the subunits of an aa3-type cytochrome oxidase localized in the inner membrane, and the rus gene encodes a periplasmatic blue copper protein, the rusticyanin.

The passage of the protons inward through the membrane results from a pH gradient (pH 2 outside, pH 5-6 inside) and occurs through channels associated specifically with adenosine triphosphatase (ATPase) on the inside of the membrane, enabling this enzyme to synthesize ATP from ADP and Pi. Thus, free energy from the oxidation of iron, which is made available to the cell, is trapped in high-energy phosphate bonds in oxidative phosphorylation of ADP with Pi to ATP.

Fig 2.5 : Genome-based models for the enzymes and electron transfer proteins involved in oxidation of (A) ferrous iron and (B) Reduced inorganic sulfur compounds (RISCs) (Valdes, 2008).



2.6 THE SULFER CYCLE

The elements like C, O, N and S are utilized as nutrients for the growth of living organisms and therefore their cycles are of prime importance (Agate 1982). Figure 2.6 & 2.7 show the cycling of sulfur and its compounds in environment and mineral deposits respectively.

There are many bacteria capable of oxidizing reduced sulfur compounds for energy. By definition, for a bacterial species to be a member of the genus *Thiobacillus*, it must be able to carry out oxidation of inorganic sulfur compounds for its energy requirements (Mwaba 1993). All strains of *A. ferrooxidans* are capable of S oxidation, although with quantitative differences noted with different strains or with the same strain grown on different substrates (Silver, 1978).

A generalized scheme for the oxidation of elemental sulfur and reduced sulfur compounds by the thiobacilli is shown in figure 2.8 (Robertson and Kuenen, 2006).

2.6.1 OXIDATION OF ELEMENTAL SULFUR

The reactions involved in the oxidation of elemental sulfur are:

$S_8 + GSH \rightarrow GS_8SH$ .....(3)

$GS_8SH + O_2 \xrightarrow{\text{Sulfur oxiding enzyme}} GS_8SO_2H$.....(4)

$GS_8SO_2H + H_2O \rightarrow GS_7SH + H_2SO_4$.....(5)

Elemental sulfur, which exists most commonly in the form of circular eight-atom molecule, is attacked by a sulfhydryl-containing agent resulting in an organic polysulphide. The sulfur oxidizing enzyme catalyses the oxidation of polysulphide, which is then removed hydrolytically as sulfite. In vitro, the sulfite is acted upon by the elemental sulfur with the formation of thiosulfate (Lundgren and Silver, 1980). In the presence of a suitable sulfite-trapping reagent, such as formaldehyde or an active sulfite oxidizing system, sulfite is shown to be the true end product of this reaction.

Fig 2.6 : The sulfur cycle in environment (Agate 1982).

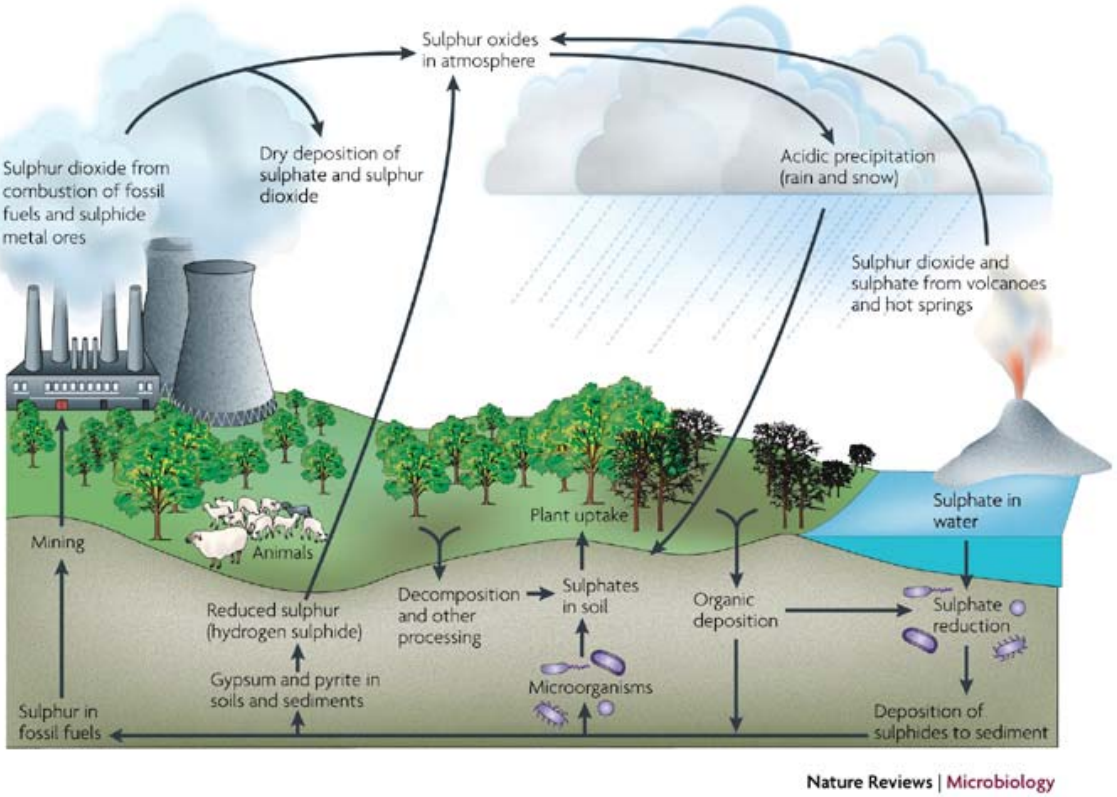


Fig 2.7 : SIMPLIFIED SULPHUR CYCLE FOR MINERAL DEPOSITS

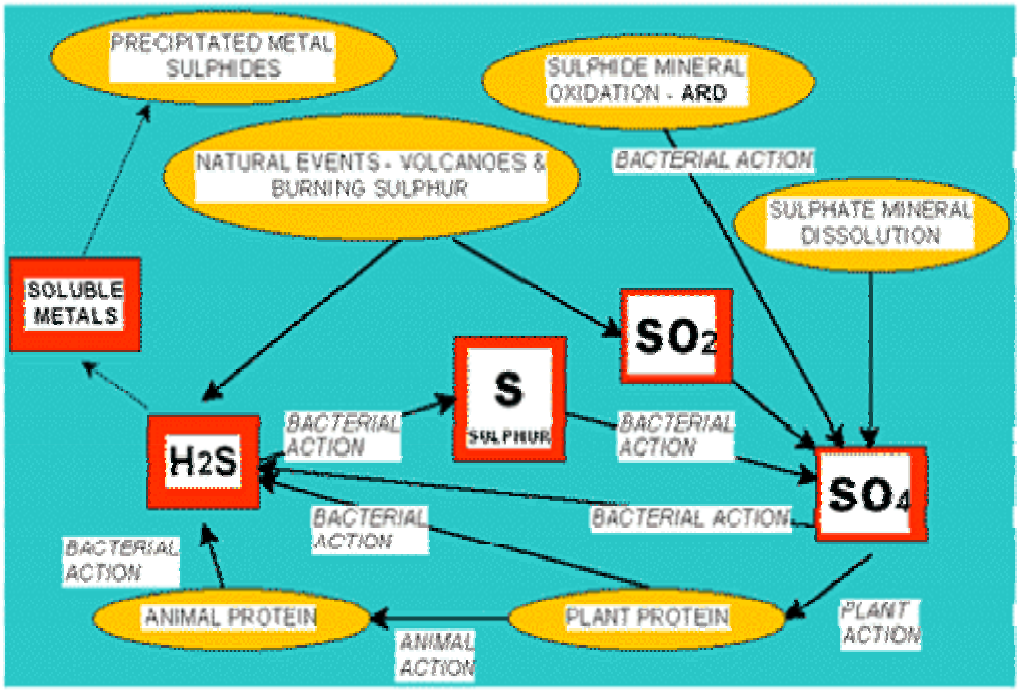
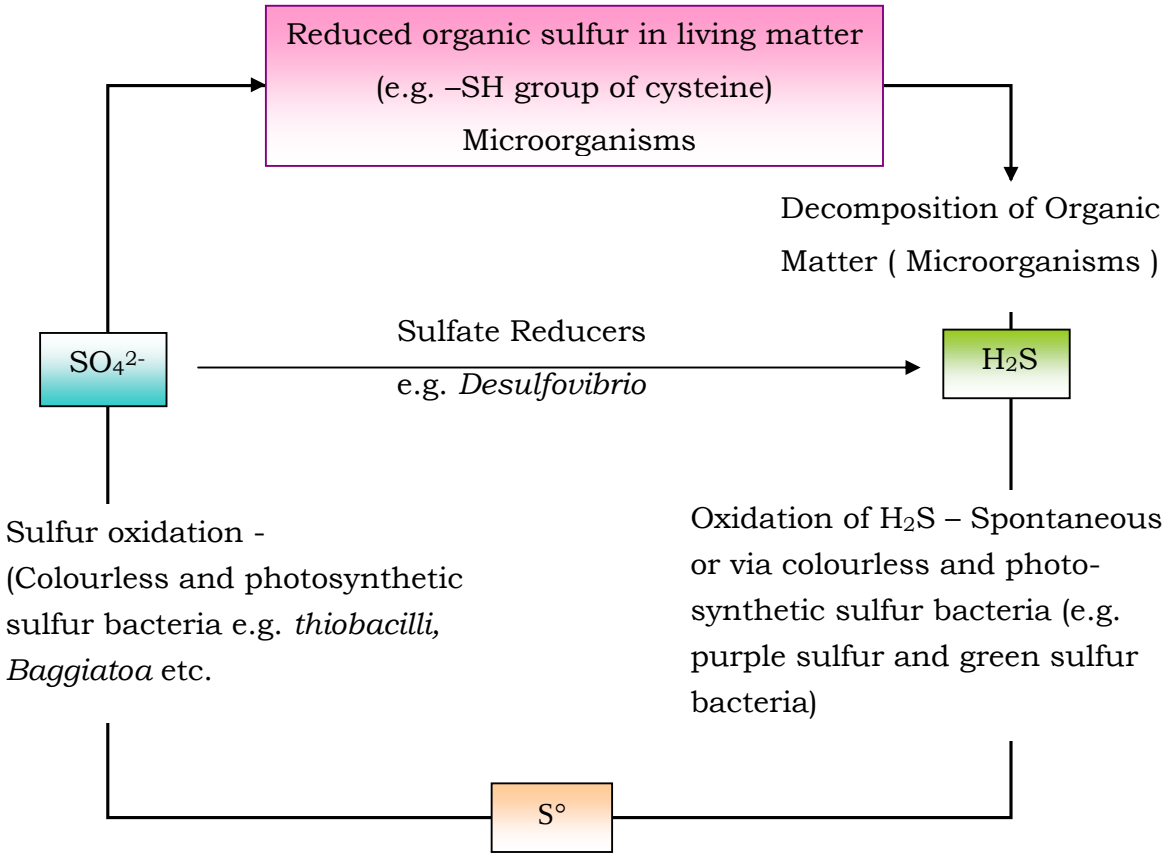


Fig 2.8 : Role of microbes in oxidation of elemental sulfur and reduced sulfur compounds (Robertson and Kuenen, 2006).



2.6.2 SULFUR OXIDISING ENZYME SYSTEMS

The iron-oxidizing bacterium *A. ferrooxidans* has enzyme systems that oxidize not only ferrous iron but also many kinds of reduced sulfur compounds such as elemental sulfur, tetrathionate, chalcopyrite and pyrite (Chandraprabha *et al.* 2002). When *A. ferrooxidans* ATCC23270 cells, grown for many generations on sulfur is grown in sulfur medium- with and without Fe^{+3}, the bacterium markedly increases not only in iron oxidase activity but also in Fe^{+2} producing sulfide:ferric iron oxidoreductase (SFORase) activity and hence they have the same ability to grow on iron as rapidly as iron-grown cells. While SFORase activity in sulfur grown cells (with Fe^{+3}) is approximately 20 times higher than that of the iron-grown cells. The results suggest that both iron oxidase and Fe^{+2} producing –SFORase have roles in the energy generation of *A. ferrooxidans* from sulfur.

2.6.3 MECHANISM OF ENERGY GENERATION IN *A. ferrooxidans* IN PRESENCE OF SULFUR

Free iron around *A. ferrooxidans* cells and /or the iron on the outer surface or in the periplasmic space of bacteria is reduced by SFORase with sulfur as an electron donor and then the Fe^{+2} produced is oxidized by iron oxidase to produce energy for cell growth and maintenance. When sulfur medium without Fe^{+3} is prepared with deionised water, but not with tap water, the growth yield may decrease or no cell growth may occur at all. Therefore, Fe^{+3} ion is absolutely necessary for *A. ferrooxidans* growth on sulfur and trace amount of Fe^{+3} ion obtained from tap water or components of sulfur medium supports the operation of both SFORase and iron oxidase (Chandraprabha *et al.*, 2002).

2.7 METAL SULFIDE OXIDATION

In biooxidation, microorganisms are used to extract metals from sulfide and/or iron containing ores and mineral concentrates. The iron and sulfide is microbially oxidized to produce ferric iron and sulfuric acid, and these chemicals convert the insoluble sulfides of metals such as copper, nickel and zinc to soluble metal sulfates which can be readily recovered from solutions (Menon and Dave, 1995; Rawlings *et al.* 2003). The sulfides of interest to mining industries are comparatively stable compounds and exist as crystalline phases in nature. A list of common metal sulfide minerals applicable to metal extraction is given in table 2.6 with the microorganism attacking it.

The biooxidation of sulfide minerals involve a primary acidic or oxidative ferric reaction with the mineral, which can be represented as: (Hansford and Vargas, 2001).

$$MS_x + Fe^{3+} \xrightarrow{\text{chemical}} M_x^{+} + Fe^{2+} + S \quad \text{.....(6)}$$

This, apart from dissolution of metal sulfide ions, produce ferrous iron and some primary sulfur compound.

Table 2.6 : Mineral sulfides oxidized and leached by microbes. (Rawlings *et al.* 2003)

Mineral sulfides	Formula	Microorganisms
Chalcopyrite	$CuFeS_2$	*A. ferrooxidans, Sulfolobus* sp.
Covellite	CuS	*A. ferrooxidans, A. thioparus*
Chalcocite	Cu_2S	*A. ferrooxidans*
Bornite	Cu_5FeS_4	*A. ferrooxidans*
Pyrite and Marcasite	FeS_2	*A. ferrooxidans, Sulfolobus* sp.
Molybdenite	MoS_2	*A. ferrooxidans*
Millerite	NiS	*A. ferrooxidans*
Cobalt sulfide	CoS	*A. ferrooxidans*
Galena	PbS	*Arthrobacter* sp.
Pyrrhotite	FeS	*Arthrobacter* sp.
Realgar	AgS	*Arthrobacter* sp. & *Hyphomicrobium* sp.
Sphalerite	ZnS	*A. ferrooxidans* and *Arthrobacter* sp.

Interactions between microorganisms and metals can be conveniently divided into 3 distinct processes, all of which may be important with respect to metal distribution (Ford and Ryan, 1995).

(a) Intracellular interaction

(b) Cell surface interaction

(c) Extracellular interaction

The biochemical fundamentals of the leaching reactions have been the subject of intensive research in recent years (Rawlings 2005). Evidences indicate that iron and sulfide can be simultaneously oxidized by bacteria and both the oxidative processes contribute to metal leaching (Agate 1982). Silverman and Ehrlich in 1964 tried to established two possible bioleaching routes for the dissolution of metal sulfides, namely: (Boon, 2001; Chandraprabha *et al.*, 2003; Rodriguez *et al.*, 2003; Zhang and Fang, 2005)

1. Direct mechanism
2. Indirect mechanism

2.7.1 Direct mechanism

For the direct oxidation mechanism, the bacteria attach themselves onto the surface of the sulfide ores and directly solubilize the surface through hypothesized enzymatic oxidation reaction (Sklodowska *et al.*, 1999; Vasan *et al.*, 2001; Zhang and Fang, 2005). The sulfur moiety of the mineral is supposed to be biologically oxidized in presence of oxygen to SO_4 and metal cations without any detectable intermediate occurring.

The following equations summerize the direct mechanism:

$FeS_2 + 3.5\ O_2 + H_2O \rightarrow Fe^{+2} + 2H^+ + 2SO_4^{-2}$.....(7)

$2Fe^{+2} + 0.5O_2 + 2H^+ \rightarrow 2Fe^{+3} + H_2O$.....(8)

2.7.2 Indirect mechanism

In contrast, the indirect mechanism basically comprises the oxidizing action of Fe^{3+} ions dissolving a metal sulfide. In the contrast of this chemical reaction, Fe^{2+} ions and elemental sulfur (S_8) shall be generated. These compounds are then biologically oxidized to Fe^{3+} ions and SO_4. This mechanism does not require the attachment of cells to the sulfide mineral.

$FeS_2 + 14Fe^{+3} + 8H_2O \rightarrow 15Fe^{+2} + 16H^+ + 2SO_4^{-2}$.....(9)

$MS + 2Fe^{+3} \rightarrow M^{+2} + S^\circ + 2Fe^{+2}$.....(10)

$S^\circ + 1.5\ O_2 + H_2O \rightarrow 2H^+ + SO_4^{-2}$.....(11)

It is now generally accepted that the originally discussed "direct mechanism" of biological metal sulfide oxidation, i.e. the direct enzymatic oxidation of the sulfur moiety of heavy metal sulfide does not exist (Agate, 1982; Rohwerder and Sand, 2007). Hence, the indirect mechanism, i.e. non-enzymatic metal sulfide oxidation by Fe^{3+} ions combined with enzymatic re-oxidation of the resulting Fe^{2+} ions remains (Agate, 1982; Fowler *et al.*, 1999; Vasan *et al.*, 2001).

The indirect mechanism comprises of sub mechanisms:

(i) The non-contact mechanism assumes that the bacteria oxidize only dissolved Fe^{2+} ions to Fe^{3+} ions. The resulting Fe^{3+} then attacks metal sulfides and after oxidizing metal sulfides gets reduced to Fe^{2+} ions (Menon and Dave, 1995). This is in effect, the previously designated indirect mechanism.

(ii) The contact mechanism requires attachment of bacteria to the sulfide surface (Natarajan and Das, 2003). In case of *A. ferrooxidans,* bacterial exopolymers contain Fe^{3+} ions, each complexed by uronic acid residues.

Mechanism of metal leaching by contact and non-contact method is diagrammatically explained in figure 2.9. Thus, the first function of complexed Fe^{3+} ions in the contact mechanism is mediation of cell attachment, while their second function is oxidation dissolution of the metal sulfide similar to the role of free Fe^{3+} ions in the non-contact mechanism.

Interfacial processes are complex interactions of electrochemical, biochemical and surface specific mechanisms (Okibe and Johnson, 2002). It is known that the attachment process is predominantly mediated by the extracellular polymeric substances (EPS) surrounding the cells and that attachment even stimulated EPS production (Escobar *et al.*, 1997). Attachment occurs due to a mainly electrostatic interaction of the positively charged cells (The ferric ions in EPS gives the cell surface a net positive charge under physiological conditions (pH 2) with negatively charged pyrite. They also play an active role in the dissolution of pyrite via an indirect mechanism.

The EPS containing complexed ferric ions comprise a reactive space, where cells interact with a pyrite surface through electro static forces and dissolution process takes place (Kinzler *et al.*, 2003). The reactive space during copper leaching is shown in figure 2.10 (Valdes 2008).

Fig 2.9 : Biooxidation of sulfide mineral by contact, non-contact and cooperative leaching method

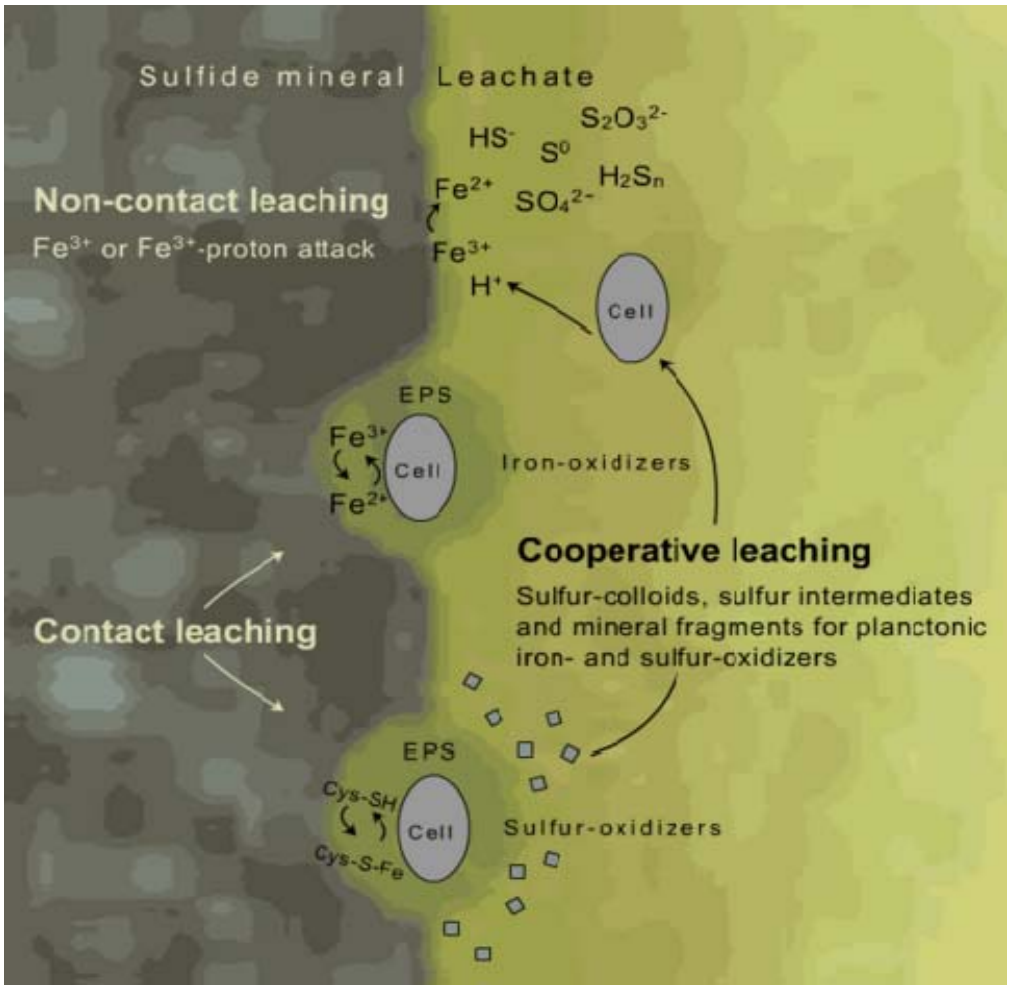
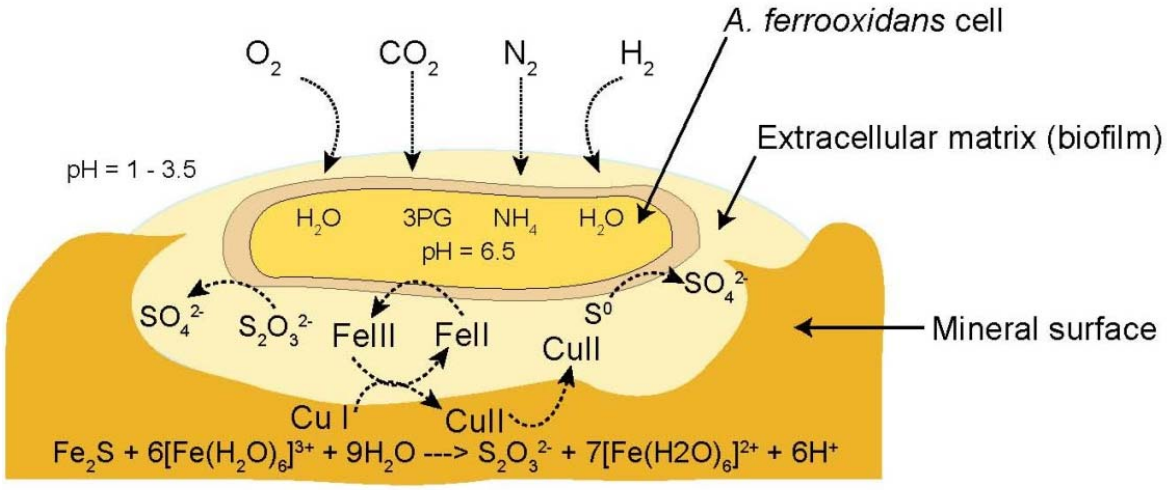


Fig 2.10 : Reactive space in EPS with Fe^{3+} iron during copper leaching



2.7.3 Galvanic conversion

The physical contact between two dissimilar metal sulfide phases, immersed in an electrolyte like dilute sulfuric acid or ferric sulfate, creates a galvanic cell. In a mixture of pyrite and chalcopyrite, the former acts as a cathode while the chalcopyrite behaves as an anode and undergoes rapid dissolution (Natarajan, 1988). *A. ferrooxidans* may accelerate the reaction by continuing oxidizing the film of elemental sulfur that would obstruct the diffusion of copper and iron salts (Http://www.oecd.org/biotrack).

2.8 METAL DISSOLUTION PATHWAYS

There are 2 indirect mechanisms in bioleaching and which one of the two pathways will take place is dependent on acid solubility of the metal sulfide (Schippers and Sand, 1999; Sand *et al.* 2001; Rohwerder and Sand 2007).

(i) Thiosulfate pathway for acid-insoluble metal sulfides such as FeS_2, which are attacked by Fe^{+3} only generating thiosulfate.

(ii) Polysulfides pathway for acid soluble metal sulfides, which are attacked by both Fe^{+3} and protons producing elemental sulfur.

2.8.1 Thiosulfate pathway for acid-insoluble metal sulfides

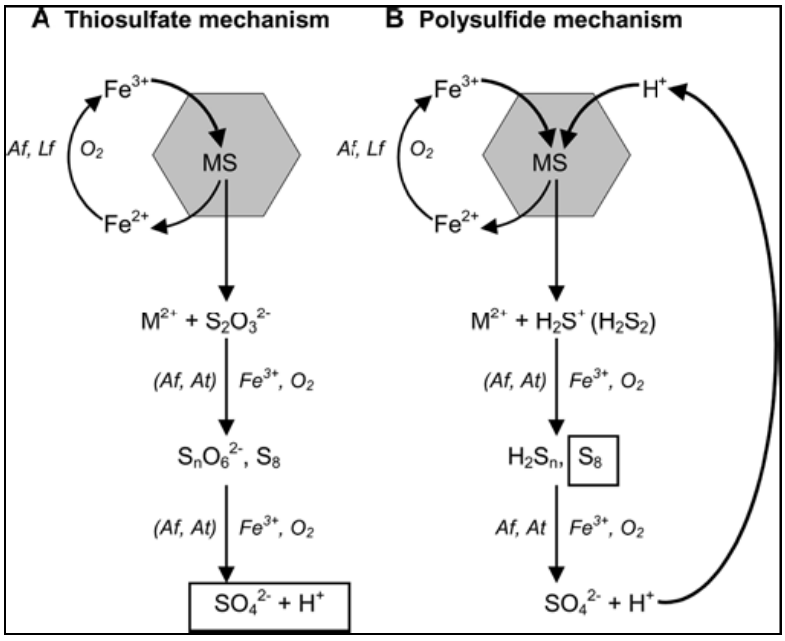
In all acid insoluble metal sulfides such as pyrite (FeS_2), molybdenite (MoS_2), and tungstenite (WS_2), the chemical bond between sulfur atom and metal ion does not break until a total of 6 successive one electron oxidation steps have been conducted and thiosulfate ($S_2O_3^{2-}$) is liberated. According to its first free sulfur compound, this mechanism is called thiosulfate pathway. Thiosulfate is further oxidized via tetrathionate, the highly reactive disulfane monosulfonic acid, and other polythionates finally to sulfate (Fig 2.11) (Rohwerder *et al.*, 2003). Since extraction of electron is carried out by Fe^{3+} ions and at a pH around 2, Fe^{2+} cannot abiotically oxidized to Fe^{3+}, only ferrous oxidizing bacteria such as *A. ferrooxidans* and *L. ferrooxidans* are able to leach acid-insoluble metal sulfides under acidic conditions.

2.8.2 Polysulfide pathway for acid soluble metal sulfides

In metal sulfides such as sphalerite (ZnS), galena (PbS), arsenopyrite (FeAsS), chalcopyrite ($CuFeS_2$) and hauerite (MnS_2), the chemical bond between metal and sulfur moiety are dissolved by a combined action of electron extraction by Fe^{3+} ions and proton attack and after binding with one proton, H_2S^+ (i.e. H_2S minus 1 electron) is liberated from the metal sulfide. This can spontaneously dimerize to a free disulfide (H_2S_2) and is further oxidized via higher polysulfides to elemental S (S_8). Consequently this mechanism was named polysulfide pathway.

In the course of polysulfide oxidation, more than 90% of the sulfide moiety of a metal sulfide is transformed to elemental sulfur in absence of sulfur oxidizing bacteria. In presence of sulfur oxidizing bacteria, free sulfide (H_2S) is oxidized via elemental sulfur to sulfuric acid and thus regenerate the protons previously consumed by the metal sulfide dissolution.

Fig 2.11 : Schematic comparison of (A) thiosulfate and (B) polysulfide mechanisms in bioleaching of metal sulfides



2.9 MINERAL RESOURCES OF INDIA

Industrial development of a country depends chiefly on its mineral resources and successful utilization of these sources. India, like other countries of the world, is not self-sufficient in her all mineral requirements. Every year, millions of foreign exchange are spent for importing necessary metals and minerals. At the same time, India occupies very prominent position in the world market for the production of some other minerals like dolomite, mica, silica, basalt, feldspar, quartz, vermiculite & natural stones like granite and marble. Table 2.7 shows the estimated reserves of important metal and minerals with the states producing them. The share of the mineral sector in the gross domestic product (GDP) of the country is ~3.5 %.

India is meeting her requirement by importing non ferrous metals like copper, zinc and lead (Parbin Singh, 1990). India accounts for about 3.5 to 4% of the world's copper production and usage. Currently, India consumes ~0.6 million tonnes of copper and it is likely to increase by 10% as the governement and private sector increasingly focus on infrastructure. India's primary copper production relies heavily upon imported concentrates, with over 0.8 million t/yr imported in 2001-02 from various countries (e.g. Chile, Indonesia, Australia, and Canada). The production capacity of refined copper in India has risen from 47,500 t in 1997 to 447,500 t in 2003 and can reach 597,500t soon (http://www.indianmetals.com/news/mineralsmining.php)

Private investment (both domestic and foreign), has been permitted for the exploration and exploitation of many minerals. As a result, several foreign companies have begun investing in India, with the majority coming from Canada and the USA, followed by Australia, the UK and South Africa. Most interest has been shown in the base metals, diamond, mineral sands and gold sectors. The leading mines in India are shown in table 2.8.

Table 2.7 : Mineral reserves in India

Metal	Recoverable Reserves (mt)	Producers of ore
Iron		Madhya Pradesh, Karnataka, Bihar, Orissa,
haematite	9,602	Goa, Maharashtra, Andhra Pradesh, Kerala,
magnetite	3,408	Rajasthan, Tamil Nadu
Lead and Zinc	199	Rajasthan, Gujarat, West Bengal, Andhra Pradesh, Uttar Pradesh, Orissa, Meghalaya, Tamil Nadu, Sikkim.
Copper	416.8	Singhbhum district (Bihar), Balaghat (Madhya Pradesh) and Jhunjunu, Alwar, (Rajasthan).
Manganese	167	Karnataka, Orissa , Madhya Pradesh, Maharshtra, Goa.
Bauxite	2,525	Orissa, Andhra Pradesh, Madhya Pradesh, Gujarat, Maharashtra, Bihar
Gold	17.79	Kolar Gold Field, Karnataka, Hutti Gold Field, Raichur, Karnataka, Ramagiri Gold Field, Anantapur, Andhra Pradesh
Coal	2,11,593	AndhraPradesh, Arunachal Pradesh, Assam, Bihar, Madhya Pradesh, Maharashtra, Meghalaya, Nagaland, Orissa, Uttar Pradesh and West Bengal
Non metallic minerals		
Asbestos	93	Karnataka, Rajasthan, Bihar
Dolomite	4,387	Madhya Pradesh, Orissa, West Bengal, Uttar Pradesh, Gujarat, Karnataka, Maharashtra.
Gypsum	238	Rajasthan, Tamil Nadu, Jammu and Kashmir,Uttar Pradesh and Gujarat.
Limestone	76,446	Andhra Pradesh, Madhya Pradesh, Uttar Pradesh, Himachal Pradesh, Gujarat, Rajasthan, Karnataka, Tamil Nadu, Maharashtra, Orissa, Bihar

(http://www.indianmetals.com/news/mineralsmining.php)

Table 2.8 : Leading mines in India

Mine	Location	Metal/ore obtained	Share Holder	Status
Agnigundala	Hyderabad	Lead	Hindustan Zinc Ltd	Underway
Chandgad	Kolhapur	Bauxite	Alcan Inc.	
Hirabudini	Karnataka	Gold	Hutti Gold Mine Company	Operating
Lohardaga mine	Jharkhand	Bauxite	Alcan Inc.	
Sargipali deposits	Sambalpur, Orissa	Lead	Hindustan Zinc Ltd	Underway
Rampura Agucha	Udaipur	Lead		Underway
Rajpura Dariba	Udaipur	Zinc	Hindustan Zinc Ltd	Underway
Tensa mines	Sundergarh Orissa	Iron Ore	Jindal Steel and Power	Operating
Zawar Mines	Udaipur	Zinc	Hindustan Zinc Ltd	Underway
Srikurmam	Andhra pradesh	Heavy Mineral Sands	Western Garnet International (74.26%)	Planned

2.10 GLOBAL METAL PRODUCTION SCENARIO

The earliest commercialization of mineral biotechnology on record is the granting of a patent to Kennecott Copper Corporation in 1958 for the use of iron oxidizing bacteria for bioleaching. In the USSR, the first pilot industrial tests were carried out at the Degtyar mine in 1964 to quantitatively evaluate the activity of *Acidithiobacillus ferrooxidans* in natural conditions and to enhance their activity at industrial level at low temperature in the range 11 – 15 °C (Mwaba, 1993).

Traditional mining countries such as the USA, Canada, Australia, South Africa and Chile dominate the global mining scene with respect to mining reserves, exploration methods and technology. World production of metals from top ten producing countries is shown in table 1.9. Exploration and development funding are now shifting to areas that have been poorly explored or have had poor access for reasons of politics, infrastructure or legislation. Gold, base metal, diamonds and platinum group elements (PGE's) are the more important commodities explored for and developed globally. Asia is a major producer of base metals, PGE's, ferrous metals and coal (http://www.mbendi.co.za/indy/ming/am/us/ p0005.htm). Various methods have evolved for maximum utilization from leaching eg. BRISA, BIOCOP and GEOCOAT methods.

2.10.1 BRISA

Among other hydrometallurgical processes, the BRISA process (Biolixiviancion Rapida Indirecta Con Separation de Acciones; Fast indirect Bioleaching with Actions Separation) has been developed and successfully applied in recent years to improve the kinetics of copper bioleaching from copper concentrates. In the BRISA process, the bioleaching process is performed in two separate stages:

(a) a chemical stage : $CuFeS_2 + 2Fe^{+3} \rightarrow 2Fe^{+2} + Cu^{+2} + 2S$……(12) and

(b) a biological stage : $2Fe^{2+} + ½\ O_2 + 2H^{+} \rightarrow 2Fe^{+3} + H_2O$…….(13)

Table 2.9 : World production of metals from top ten producing countries with estimated reserves left

Metal	Production	Reserves	Reserve Base	Reserve life	Reserve Base Life
	(X 10^6) Metric ton				
Aluminium (Bauxite)	31.9	Large	Large	>100 years	> 100 years
Iron	1380	72,000	164,000	52.2	>100 years
Copper	15	430	850	28.7	56.7
Zinc	10	200	420	20.0	42.0
Lead	3.3	61	130	18.5	39.4
Nickel	1.4	56	130	40.0	92.9
Molybdenum	0.18	7.8	17	43.3	94.4
Tin	0.3	5.5	10	18.3	33.3
Silver	0.019	0.245	0.52	12.3	26.1
Platinum and Palladium	0.000206+ 0.000214	0.065	0.073	>100 years	>100 years
Gold	0.002519	0.038	0.082	15.1	32.6

This biological stage is responsible for the biooxidation of ferrous iron consumed in the chemical stage. To improve chalcopyrite leaching rate, silver ion is used in ferric leaching circuit. Since chemical and bacterial actions are separated; the silver does not come in contact with bioleaching reactor (Romero et al 2003).

2.10.2 BIOX® and BioCOP process

The first bioleach laboratory tests of BIOX® process were performed in the seventies and possibly even before. BHP Billiton's Johannesburg Technology Centre (JTC), then known as Genmin Process Research, was involved with subsequent development and commercialization of BIOX® process. This process utilized mesophilic microorganisms (±40°C) to oxidize sulphide minerals (mainly pyrite pyrrhotite and arsenopyrite) to liberate gold in refractory sulfide gold concentrates (Budden and Spenser, 1993).

The leaching was carried out in stirred reactors with oxygen supplied through air injection and temperature controlled by immersed cooling coils. By 1994-95 commercial units BIOX® were made with 1000 m^3 reactor operating volumes and new BIOX plants are still continually being built with Goldfield Ltd. having the license for BIOX® plants. Table 2.10 gives a summary of the commercial BIOX plant, previously or currently in operation (Arrascue and Niekerk, 2006; Van Aswegen *et al.*, 2007).

The biomining industry has a long-standing interest in the use of extreme acidophiles for metals recovery from ores. In case of chalcopyrite the oxidation stops at 30%-60% oxidation and higher operation temperature using thermophiles and mesophiles has desired impact on copper dissolution (Siezen and Wilson, 2009). In chalcopyrite oxidation, final dissolution effectively doubled from 45% to +90% through the use of thermophiles (Norris *et al.*, 1980; Lindstrom *et al.*, 1992; Munoz et al 2006). Hence, BIOX® technology was modified using thermophilic microorganisms (75°C) and BioCOP™ process was developed by alliance of BHP Billiton and Codelco mine of Chuquicamata, Chile and this joint venture company is called Alliance Copper Limited. Maturation of the BIOX® process for tank bioleaching of gold and the BioCOP™ process for tank bioleaching of copper is shown in figure 2.12 (Clark *et al.*, 2006).

Fig 2.12: Development and upgradation of BIOX® into BioCOP™ process

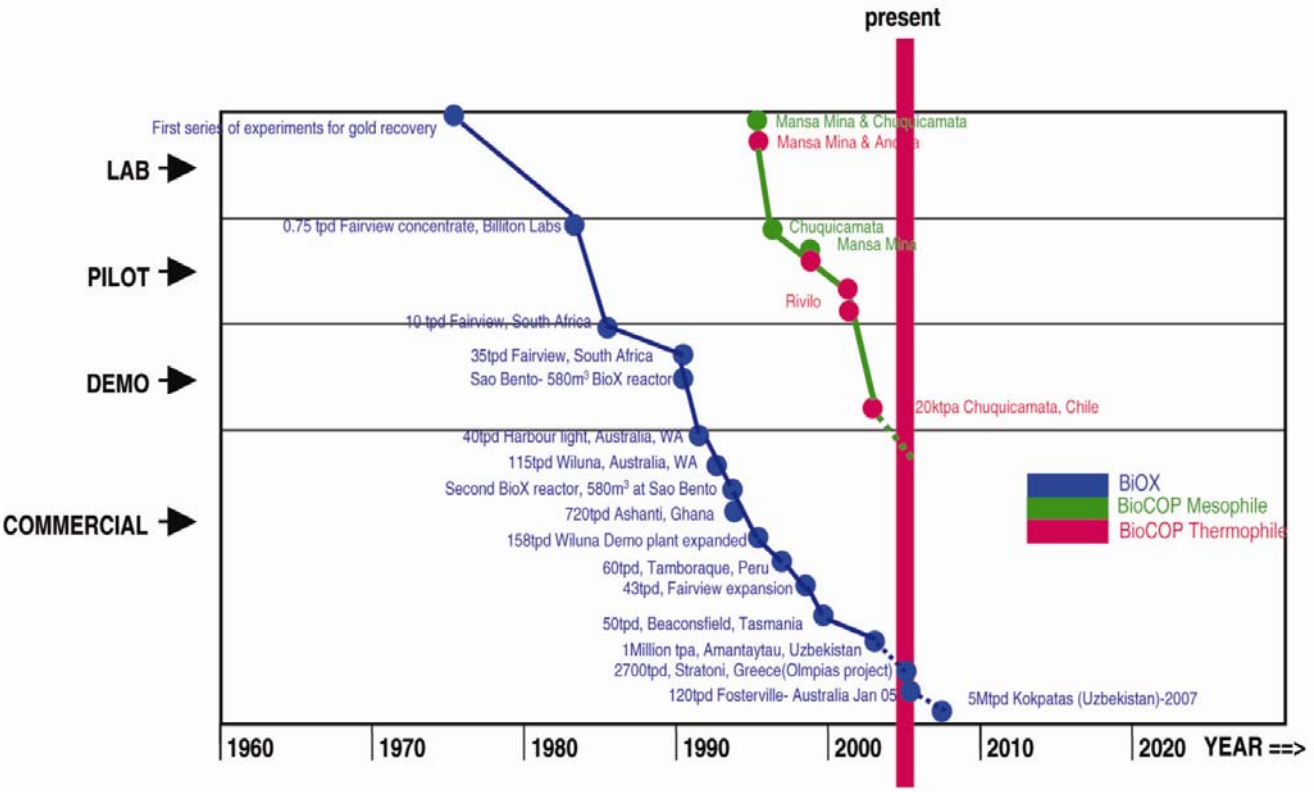


Table 2.10 : Current and previous commercial BIOX operations.

Mine	Country	Concentrate treatment capacity [ton/day]	Reactor size [m³]	Date of commissioning
Fairview	South Africa	62	340	1986
Sao Bento	Brazil	150	550	1990
Harbour Lights	Australia	40	160	1991
Wiluna	Australia	158	480	1993
Sansu	Ghana	960	900	1994
Tamboraque	Peru	60	262	1998
Fosterville	Australia	211	900	2005
Suzdal	Kazakhstan	196	650	2005

BioCOP™ technology is employed by alliance of BHP Billiton and Codelco mine of Chuquicamata, Chile and this joint venture company is called Alliance Copper Limited. For this technique, the first thermophile trials on copper concentrate began in1995 and pilot plant was started in 1997. The full scale commercial demonstration plant with a production rate of 20,000 tons copper per annum was commissioned in August 2003 (Batty and Rorke 2006)

2.10.3 GEOCOAT

Tank biooxidation is successful method for achieving high metal recoveries, but both capital and operating costs are relatively high. Heap biooxidation has lower cost but suffers from low metal extraction rates and low ultimate metal recoveries. GeoBiotics has developed and patented GEOCOAT® biooxidation technology for treatment of sulfide base metals and gold concentrates, which offers a unique approach of bacterial mineral processing, combining the low capital and operating costs of heap leaching with the high recoveries obtained in agitated tank bioreactors (Harvey and Bath 2007).

In this process, thickened floatation concentrate is applied on heap of gravel-sized support rock, forming a thin adherent concentrate coating on the support rock particles. The stacked heap has an open structure and is highly permeable to flows of solution and air. Acid solution, which contains acidophilic bacteria, is introduced using a sprayer system and circulated through the heap to bioleach the contained metals. The inoculum used was a mixed mesophilic culture containing *A.ferrooxidans, A.thiooxidans, L.ferrooxidans*. Final zinc dissolution after 90 days can be upto 91% with a corresponding sulfide oxidation of 89%. (Gehrke *et al.*, 1998; Coram-Uliana *et al.*, 2006). GeoBiotics LLC and Kumba Resources (Pvt) Ltd. are investigating the feasibility of applying the GEOCOAT® process to the leaching and recovery of zinc from a low-grade sphalerite concentrate produced from the accumulation of floatation tailings by Kumba's Rosh Pinah zinc mine in Namibia (Coram-Uliana *et al.*, 2006; Viera et al, 2007).

Table 2.1 : Physiological property of metal sulfide oxidizing, acidophilic microorganisms. (Johnson et al., 2005; Schippers, 2007)

Species#	pH		Temp (°C)		Pyrite	Other MS	Fe^{+2} ions	Sulfur	Growth
	Optimum	Range	Optimum	Range					
Mesophilic and moderately thermophilic bacteria									
Acidimicrobium ferrooxidans	~2	NA	45-50	<30-55	+	NA	+	-	F
Acidithiobacillus albertensis	3.5-4.0	2.0-4.5	25-30	NA	-	+	-	+	A
Acidithiobacillus caldus	2.0-2.5	1.0-3.5	45	32-52	-	+	-	+	F
Acidithiobacillus ferrooxidans	2.5	1.3-4.5	30-35	10-37	+	+	+	+	A
Acidithiobacillus thiooxidans	2.0-3.0	0.5-5.5	28-30	10-37	-	+	-	+	A
Alicyclobacillus disulfidooxidans	1.5-2.5	0.5-6.0	35	4-40	+	NA	+	+	F
Alicyclobacillus tolerans	2.5-2.7	1.5-5	37-42	<20-55	+	+	+	+	F
"Caldibacillus ferrivorus"	1.8	NA	45	<35->55	+	NA	+	+	F
"Ferrimicrobium acidiphilum"	2-2.5	1.3-4.8	37	<10-45	+	NA	+	-	H
Leptospirillum ferriphilum	1.3-1.8	NA	30-37	NA-45	+	+	+	-	A
"Leptospirillum ferrodiazotrophum"	NA	<1.2<	NA	<37<	NA	NA	+	NA	A
Leptospirillum ferrooxidans	1.5-3.0	1.3-4.0	28-30	NA	+	+	+	-	A
Sulfobacillus acidophilus	~2	NA	45-50	<30-55	+	+	+	+	F
"Sulfobacillus montserratensis"	1.6	0.7->2	37	<30-45	+	NA	+	+	F
Sulfobacillus sibiricus	2.2-2.5	1.1-3.5	55	17-60	+	+	+	+	F
Sulfobacillus thermosulfidooxidans	~2	1.5-5.5	45-48	20-60	+	+	+	+	F
Sulfobacillus thermotolerans	2-2.5	1.2-5	40	20-60	+	+	+	+	F

Species#	pH		Temp (°C)		Pyrite	Other MS	Fe^{2+} ions	Sulfur	Growth
	Optimum	Range	Optimum	Range					
"Thiobacillus plumbophilus"	NA	4.0-6.5	27	9-41	-	+	-	+	A
"Thiobacillus prosperus"	~2	1.0-4.5	33-37	23-41	+	+	+	+	A
Thiomonas cuprina	3.5-4	1.5-7.2	30-36	20-45	-	+	-	+	F
Mesophilic and moderately thermophilic archaea									
"Ferroplasma acidarmanus"	1.2	<0-1.5	42	23-46	+	NA	+	-	F
Ferroplasma acidiphilum	1.7	1.3-2.2	35	15-45	+	NA	+	-	F
"Ferroplasma cupricumulans"	1-1.2	0.4-1.8	54	22-63	NA	+	+	+	F
Extremely thermophilic archaea									
Acidianus brierleyi	1.5-2.0	1-6	~70	45-75	+	+	+	+	F
Acidianus infernus	~2	1-5.5	~90	65-96	+	+	+	+	A
Metallosphaera hakonensis	3	1-4	70	50-80	NA	+	NA	+	F
Metallosphaera prunae	2-3	1-4.5	~75	55-80	+	+	+	+	F
Metallosphaera sedula	2-3	1-4.5	75	50-80	+	+	+	+	F
Sulfolobus metallicus	2-3	1-4.5	65	50-75	+	+	+	+	A
Sulfolobus yangmingensis	4	2-6	80	65-95	NA	+	NA	+	F
Sulfurococcus mirabilis	2-2.6	1-5.8	70-75	50-86	+	+	+	+	F
Sulfurococcus yellowstonensis	2-2.6	1-5.8	60	40-80	+	+	+	+	F

Listed in alphabetical order; NA - data not available; MS – metal sulfide; A – Aerobic; F- Fermentative

Species without standing in nomenclature are given in quotation marks

Table 2.3 : Key features of the type strains of all the 8 species belonging to genera *Acidithiobacillus, Halothiobacillus and Thermithiobacillus.* (Schippers, 2007)

Organism	**Culture collection no.**	**Nucleotide sequence accession no.**	**DNA G+C content (mol%)**	**Conditions for optimum growth (Limits in parenthesis)**			**Lowest pH after growth on sulfur comp.**	**Other special properties**
				Temp (°C)	pH	NaCl (M)		
Acidithiobacillus thiooxidans	ATCC 19377^T NCIB 8347^T	M79396-8 M79401	52	28-30 (10-37)	2.0-3.0 (0.5-5.5)	0	0.5-0.8	
Acidithiobacillus ferrooxidans	ATCC 23270^T	M79404-6	58-59	30-35 (10-37)	2.5 (1.3-4.5)	0	1.5	Growth with Fe^{2+} as sole energy source
Acidithiobacillus caldus	DSM 8584^T	Z29975	63.1-63.9	45 (32-52)	2.0-2.5 (1.0-3.5)	0	NA	
Acidithiobacillus albertensis	ATCC 35403^T	NA	61.5	25-30	3.5-4.0 (2.0-4.5)	0	2.0	Glycocalyx and tuft of flagella
*Halothiobacillus neopolitanus**	NCIB 8539^T	M79399 M79419-20	56	28-32 (8-39)	6.5-6.9 (4.5-8.5)	ST$^{\#}$	2.8-3.3	
Halothiobacillus halophilus	DSM 6132^T ATCC 49870^T	U58020	64.2	30-32 (26-36)	7.0-7.3 (6.5-8.4)	1.0 (4.0)	5.6-6.0	Obligate halophile
Halothiobacillus hydrothermalis	DSM 7121^T	M90662	67.1-67.4	35-40 (11-48)	7.5-8.0 (5.5-9.0)	0.43 (0-2.0)	4.8	Poor growth without NaCl
Thermithiobacillus tepidarius	DSM 3134^T ATCC 43215^T	M79424-5	66.6	43-45 (20-52)	6.8-7.5 (5.5-8.0)	0	4.5-5.0	

On the basis of the literature survey it is concluded that the microorganism have inherent metabolic activity as well as genetic structure on the basis of the ecological site and even the microenvironment of the location from which it is isolated. Moreover, if the site of isolation has presence of heavy metal, metalloid and other ions, the isolate might be tolerant to these respective ions *in vitro* also.

Hence, the research work was undertaken to study different biomining bacteria isolated from various mines across India, in order to ascertain their specific requirement and conditions suitable for its adaptation in biohydrometallurgical process.

The research work was designed with following aims:

1. Culturing of metallurgically important bacteria and finding its preferential growth on ferrous, sulfur and thiosulfate.
2. Study of metabolic characteristics of the selected isolates on different energy sources including inorganic and organic substrates and metal sulphides.
3. Study of mixotrophy in iron oxidizing isolates.
4. Study of tolerance against metal, metalloids and ferric ions .
5. Molecular characterization and identification of selected isolates.
6. Differentiating the isolates on the basis of phylogenetic analysis of 16S rRNA fragments and random amplified polymorphic DNA (RAPD) analysis.
7. Development of selected cultures for biohydrometallurgical processes.
8. Biohydrometallurgical applications of the selected organisms for specific ore, concentrate or tailing.

4.1 INTRODUCTION

Acidithiobacillus ferrooxidans (previously *Thiobacillus ferrooxidans*) was first isolated by Colmer *et al.* (1950). It was named and characterized as a chemolithotroph by Temple and Colmer (1951). It has derived its name from Latin noun *ferrum,* which means "iron" and *oxidans* means oxidized/ made acid. It is gram negative, strictly aerobic, motile rod (0.5-1.0 μm) occurring usually single or in pairs. A number of isolates regarded as strain of *A. ferrooxidans* have been reported as having polar or peritrichous flagella and/or having pili (Kelly and Harrison, 1989).

A. ferrooxidans has been classified primarily on the basis of its ability to utilize ferrous iron as its sole energy source and fixing atmospheric CO_2 as its sole carbon source (Kelly and Wood, 2000; Robertson and Kuenen, 2006). However this organism can also derive its energy by oxidation of reduced sulphur compounds like elemental sulphur, tetrathionate, thiosulphate, thiocynate, pyrite and numerous sulphide minerals (Eccleston, 1978; Rawlings and Kusano, 1994; Sugio *et al.* 2007).

The G+C% of type strain of *A. ferrooxidans* (ATCC 23270) is 58-59. The type strain is a typical obligately chemolithotrophic *Acidithiobacillus*, deriving energy from oxidation of Fe^{2+} iron, sulphur and thiosulphate. This can also obtain energy from oxidizing Cu^{+} to Cu^{2+} and Se^{2+} to Se^{0} and from the oxidation of antimony compounds. The type strain is motile by a single polar flagellum.

On solid media (agar or agarose) with low concentration of ferrous sulphate, microscopic colonies are formed giving amber zone in the medium around them. With higher iron concentration, round colonies (1mm) are produced that become rust coloured and hard with deposited ferric salts. When grown on thiosulphate or tetrathionate agar, *A. ferrooxidans* form small (0.5-1.0 mm), round, sometimes with irregular margins and white colonies with sulphur deposition. In liquid medium containing ferrous sulphate, all species of acidophilic iron oxidizers including *A. ferrooxidans* oxidize ferrous iron to ferric iron and change

the colour of medium from a clear pale green to an amber to a red-brown with ferric sulphate. At pH 2.2 and above, considerable precipitation and encrustation of basic ferric sulphates (jarosites) take place.

Most strains of *A. ferrooxidans* are reported to fix atmospheric nitrogen (Stevens *et al.*, 1986). At least 15 strains of *A. ferrooxidans* were reported to contain *nifHDK* genes for the enzyme nitrogenase (Rawlings, 2005). Ammonium salts and probably nitrate can be used as nitrogen source. Tuovinen *et al.* (1971) mentioned that NH_4 concentration of 0.2 mM is sufficient to satisfy the nitrogen requirement of *A. ferrooxidans*. It grows better on NH_3-N than on NO_3-N. It can also use the amino acids alanine, glutamic acid, lysine, arginine or histidine to satisfy its complete nitrogen requirements. Rojas-Chapana and Tributsch (2000) reported that addition of small amount of amino acid cysteine to acidic solution enhanced the pyrite bio-oxidation rate by three times.

Many scientists have reported facultative autotrophic or mixotrophic nature of *A. ferrooxidans* strains (Shafia *et al.*, 1969; Tabita and Lundgren, 1971). But afterwards it was considered to occur due to presence of heterotrophic/mixotrophic contaminants (Harrison *et al.* 1980) and this organism is now placed as obligate autotroph in recent reclassification by Kelly and Wood (2000) and later on by Robertson and Kuenen (2006). However there have been many reports that many organic substances are beneficial to *A. ferrooxidans* and promote better growth (Shafia *et al.*, 1969; Tabita and Lundgren, 1971; Patel, 2009).

Silver *et al.* (1967) reported that presence of 5% to 10% glucose inhibited iron and sulphur oxidation by *Ferrobacillus ferrooxidans* (now *Acidithiobacillus ferrooxidans)* upto 49% (Kelly and Tuovinen, 1972; Kelly and Wood, 2000). On contrary to that, Shafia *et al.* (1969, 1972) and Tabita and Lundgren (1971) reported that following a brief adaptation period to glucose, *A. ferrooxidans* (syn. *Ferrobacillus ferrooxidans*) was grown heterotrophically on glucose, mannitol, several other sugars and a few amino acids in the absence of an oxidizable iron source. While Tuttle

and Dugan (1977) and Onysko *et al.* (1984) showed that in presence of organic acids like propionic, butyric, valeric, hexanoic, oxalacetic, benzoic and sorbic acids, *A. ferrooxidans* cells ruptured and released intracellular substances in the medium. These acids also retarded the iron oxidation by the cells.

A. ferrooxidans is acidophilic having optimum pH in the range of 1.5 to 2.5 pH. However growth is observed in the range of pH 1.3-4.5. Even though the external pH of the environment in which extreme acidophiles such as biomining microbes grow is low (e.g pH 1.0-2.0), the internal cellular pH remains close to neutral (Cox *et al.,* 1979). The difference results in a steep pH gradient across the cell membrane, which is important for nutritional purpose specially when a weak reductant such as ferrous iron is used as an electron donor (Rawlings, 2005).

Optimum temperature for growth of *A. ferrooxidans* is 30°C -35°C, though growth is observed in range of 10°C -37°C. It grows best in an aerobic environment with oxygen as an electron acceptor. If oxygen is not available and reduced sulphur compounds or formate is used as electron donor, ferric ions can serve as electron acceptor. There are also report of three strains of *A. ferrooxidans* including the type strain ATCC 23270, growing in basal salt medium using H_2 as sole energy source.

Most strains of *A. ferrooxidans* contain Q-8 type of ubiquinone, while ATCC 19859 also contains a small amount of Q-9. Major β-hydroxy acid present in *A. ferrooxidans* is 3-hydroxy tetra decanoic acid (3-OH 14:0) and major nonhydroxylated fatty acid is 18:1+19 cyc, which means that 18 carbon chain long fatty acid is present having 1 double bond and it contains 19 cyclopropane fatty acids (Kelly and Harrison, 1989).

Because of the difference of redox potential between the Fe^{2+}/Fe^{3+} and O_2/H_2O, redox couple is small and because only 1 mole of electron is released per mole of iron oxidized, vast amount of ferrous iron need to be oxidized to produce relatively little cell mass (Rawlings, 2005). The potential amount of energy made available when a sulphur atom from a

sulphide ore is oxidized to sulphate is much greater than when iron is oxidized (Pronk *et al.*, 1990).

In addition to its unique physiology, *A. ferrooxidans* also has inherent resistance to high concentrations of metallic and other ions. *A. ferrooxidans* is particularly prevalent in acid drainage waters present in mines of sulphide minerals, mineral leach dumps and drainage waters from coal mines and coal or spoil heaps (Agate, 1982).

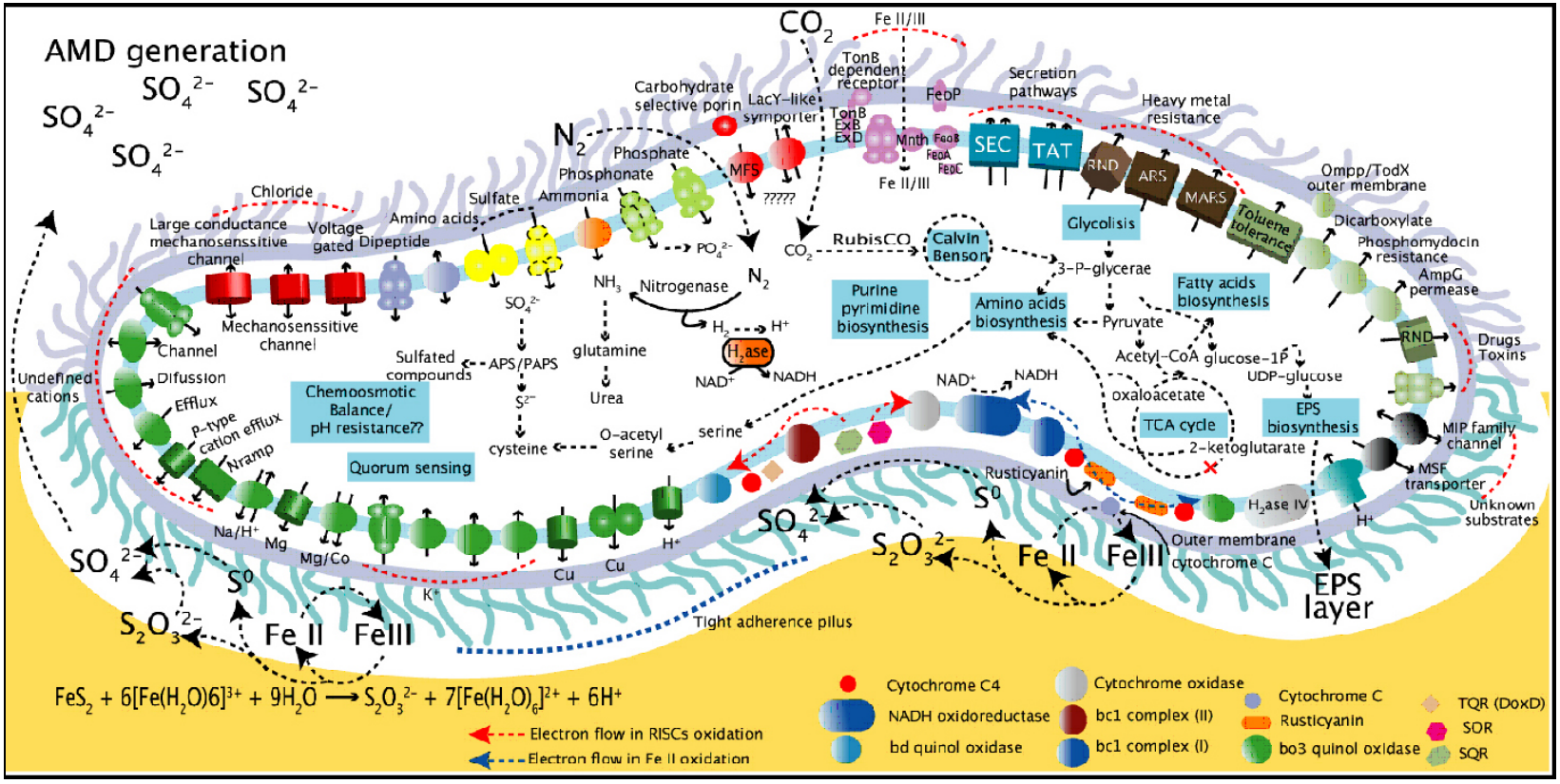
A comprehensive genome-based model of cellular metabolism of *A. ferrooxidans* ATCC 23270 is given in fig. 4.1, showing predicted transport systems, chemolithoautotrophic components, carbon, nitrogen and sulphur metabolism and biogeochemical cycling (Valdes, 2008). In the present study, we have examined the effect of several organic carbon sources on growth and iron oxidation along with utilization of conventional energy sources like thiosulphate, tetrathionate, sulphur, pyrite and thiocynate.

4.2 MATERIALS AND METHODS

4.2.1 ORGANISM

Two hundred fifteen strains of ferrous iron oxidizer isolated from soil and water sample of various mines across India were preserved in Department of Microbiology, School of Sciences, Gujarat University, Ahmedabad. The cultures were isolated by various researchers during 1991 to 2006 and preserved at 4°C in sterile acidified pH 2 distilled water added with 1% $FeSO_4.7H_2O$. Out of them, fifty one isolates were reactivated and screened. On the basis of screening by ferrous oxidation activity, eight isolates were selected for the study. Culture SRD 5 was isolated as the dominant culture from the leachate of packed bed column developed for leaching of multimetal ore from Ambamata polymetallic mine, Ambaji, Gujarat. Culture E' was isolated from Ambamata polymetallic mine. Out of the eight cultures, four cultures HGM 17, HGM 26, HGM 30 and HGM 38 were isolated by S.R. Dave and D.R. Tipre from Hutti Gold mine, Raichur district, Karnataka.

Figure 4.1: Whole-cell model of cellular metabolism of *A. ferrooxidans* ATCC 23270: Genome-based model of the cellular metabolism of *A. ferrooxidans* including predicted transport systems; chemolithoautotrophic components; carbon, nitrogen and sulfur metabolism; and biogeochemical cycling (Valdes, 2008).



Culture 2MC was isolated from Malanjkhand copper mine, Madhya Pradesh and culture Lig-tf was isolated from lignite mine drainage by S.R. Dave and A.G. Menon (Dave *et al.*, 1995; Menon and Dave, 1996).

4.2.2 GROWTH MEDIUM

Inorganic medium given by Silverman and Lundgren (1959) (9K medium) was modified in terms of ferrous sulphate concentration and prepared as shown in appendix 1. Working system of 100 ml in 250 ml Erlenmeyer flasks were used in all the experiments if not mentioned otherwise. Ferrous sulphate was used as sole energy source and it was sterilized by exposing to 256 nm weavelength UV light at a distance of 15 cm for 10 min. and added separately in the sterile medium as 0.22 μm membrane filtered solution.

4.2.3 GROWTH OF IRON OXIDIZING CULTURES

Flasks containing sterile 9K medium were inoculated with 5% (v/v) preserved cultures and incubated at Newtronics Environmental Orbital Shaker agitating at 125 rpm and adjusted at 30±2°C temperature. Flasks were regularly checked for growth which was monitored on the basis of ferrous sulphate oxidation, turbidity, microscopic observation for increase in cell number and change in colouration of the medium.

The flasks showing growth of organism were taken and culture was further enriched to ensure maximum growth. For this 10 ml aliquote of the flask was transferred as inoculum in fresh flask having 90 ml medium to increase growth of activated bacterial cultures. In 3rd sub-culturing, the culture attained 95% oxidation of added ferrous sulphate. From this flask, culture was harvested and preserved by the following method described by Dave (Dave, 1980).

The isolates were grown on the solid medium devised by DB Johnson (DBJ medium) to study the colony morphology of each isolate (Johnson *et al.* 1987; Johnson and McGinness, 1991). DBJ medium was prepared containing <0.1% ferrous sulphate. Medium was solidified using 1% agarose. After inoculation the plates were incubated at 30 °C for 21 days.

4.2.4 PURIFICATION OF THE CULTURE

Since the cultures were obtained from mine water/soil samples/coal mines/ leach columns and preserved in liquid medium, obtaining a pure monoculture was necessary for characterization and further study. Hence, all the cultures were purified by serial dilution method as given in appendix II.

4.2.5 PRESERVATION

Actively grown culture were harvested by membrane filtration using 0.22 µm Nylon filters (Pall lifesciences, India) and were washed with sterile acidified distilled water to remove remaining ferric. Liquid samples were then preserved in sterile stoppered plastic vials under refrigeration.

4.2.6 SUBSTRATE KINETIC STUDIES

Each harvested pellet was washed by resuspension in acidified sterile, pH 1.8 distilled water and centrifuged to get ferric free culture pellet. The pellets were again resuspended in acidified sterile, pH 1.8 distilled water to make inoculum and its cell load was adjusted to contain 10^8 cells/ml.

For growth kinetic experiments 10 ml of above mentioned inoculums were inoculated into 250 ml Erlenmeyer flasks containing 90 ml 9K medium, modified in terms of ferrous sulphate concentration. Ferrous sulphate ($FeSO_4.7H_2O$) was added as sole energy source and its concentration in different flasks varied from 0.25 to 12% (w/v). The flasks were placed on rotary shaker and were maintained at 30°C for the duration of each experiment. Small samples were aseptically removed from each flask just after inoculation (0 h) and at regular intervals thereafter. Ferrous present in the samples was measured by titration of 1 ml aliquot with 0.01 N potassium dichromate in presence of 5ml acid mixture using diphenylamine as indicator. Substrate utilization profile was plotted as per Lizama and Suzuki (1989), to determine V_{max} and K_m values.

Triplicate flasks were used so that a mean and standard error could be calculated for the measured quantities at each sampling time.

4.2.7 EXAMINATION OF THE ISOLATES BY SCANNING ELECTRONE MICROSCOPY (SEM)

Cells in their logarithmic growth phase were harvested as per appendix 2 and the bacterial pellet was washed thrice using acidified sterile, pH 1.8 distilled water. Finally the washed pellets were resuspended in 100 µl acidified sterile distilled water. Smear from the resuspended pellet was prepared on a grease free glass slide and allowed to air dry. The slide was further mounted on ion spluttering device and 10 mA current was passed for 2 mins. By this method slide was coated with 300ºA thickness of gold along with mounted culture. The slide was observed by scanning electron microscope model Leo 440i and scanned at different magnifications to observe the morphology of isolated strain.

4.2.8 GROWTH PROFILE OF THE CULTURES ON DIFFERENT ENERGY SOURCES

To study the metabolic difference among isolates, different types of media were prepared. Basal salt medium S° was prepared as per Bergey's manual (Kelly and Harrison, 1989). Ninety ml of basal salt medium of pH 2.7 supplemented with 1% (w/v) ferrous sulfate was taken in 250 ml Erlenmeyer flask. For pyrite utilization study, 1% pyrite of -200+325 mesh size was added in 90 ml basal salt medium of pH 1.9. For growth on sulphur, basal salt medium S° was supplemented with 1% elemental sulfur and its pH was set at 3.5. To check growth on thiosulfate, 1% and 6% sodium thiosulfate was added in the basal salt medium and pH was adjusted to 4.6. Tetrathionate was added at 1% concentration in basal salt medium. For growth on thiocynate, 0.2% ammonium thiocynate was added in the basal medium. To check the growth of organism on bicarbonate or nitrate as sole energy source, 0.05% sodium bicarbonate or 0.5% potassium nitrate was added in the basal salt medium. The screw cap test tubes having sodium bicarbonate and potassium nitrate

were filled upto the rim and stoppered to observe growth in anaerobic condition. One flask each having 2% ferrous sulfate as energy source were supplemented with 4% KH_2PO_4 and 5 % sodium chloride to check the effect of higher amount of phosphate salt or NaCl on ferrous iron oxidation activity.

Three metal sulfides viz. copper sulfide (CuS), nickel sulfide (NiS) and zinc sulfide (ZnS) were added individually in 1% (w/v) concentration in the basal salt medium. Five organic substrates like methanol, formaldehyde, sodium formate, methyl amine and methyl formate were also added in 1% w/v concentration to check their ability to serve as sole energy source for *A. ferrooxidans*.

As shown above, three sets of each media were prepared. All the chemicals used in this study were AR grade. Each medium was prepared in 250 ml Erlenmeyer flasks having 90 ml working volume that was inoculated with 10% (v/v) actively growing inoculum having cell load of 1.5 X 10^8 cells/ml. All the flasks were incubated on environmental shaker at 30 ± 2 °C at 150 rpm and results were noted at intervals of 24 h.

In ferrous sulphate and pyrite containing medium, ferrous iron was measured every 24 hours and readings were noted till 99% iron oxidation was completed. Ferrous iron oxidation was monitored by titrimetric method using 0.01 N potassium dichromate (AR grade) using diphenylamine as an indicator (Vogel, 1961). Growth on sulphur medium was ascertained on the basis of pH drop and estimation of sulphate formed in the medium. Utilization of thiosulfate was observed by estimation of thiosulfate in the medium by titrating with 0.01N iodine using 1% starch as an indicator as given in appendix II (Vogel, 1961). In flasks with different energy sources, bacterial growth was monitored every 24 h by counting the number of viable cells from supernatant under high power lenses (45X) of compound microscope using Petroff Housser counter.

4.2.9 MIXOTROPHIC GROWTH OF *Acidithiobacillus ferrooxidans* IN PRESENCE OF ORGANIC CARBON SOURCES

To study the mixotrophic growth of *A. ferrooxidans* modified Silverman and Lundgren (9K) medium (1959) was used with 3% ferrous sulphate concentration. Medium pH was set at 2.5 to maintain balance between iron oxidation as well as minimize sugar decomposition. Each carbon source was sterilized separately in 10 ml 7.0 pH distilled water and added in sterile medium before inoculation so as to give 0.5% final carbon source concentration. Medium was inoculated with 10% v/v actively growing inoculum. All the flasks were incubated on environmental shaker at 30 ± 2 °C at 150 rpm. After regular intervals sample was withdrawn and remaining ferrous iron was measured by titrimetric method. Growth was measured in terms of optical density at 520 nm after adding $1/5^{th}$ volume of 10% H_2SO_4 in the sample to decolorize any ferric pigmentation.

4.3 RESULT AND DISCUSSION

4.3.1 GROWTH OF ORGANISM AND SUBSTRATE KINETIC STUDIES

The eight iron oxidizing isolates HGM 17, HGM 26, HGM 30 and HGM 38 (Hutti gold mine, Karnataka); Lig Tf (lignite sample from Neyveli lignite mine); 2MC (Malanjkhand copper mine); SRD 5 and E' (Ambamata multimetal mine) were grown on ferrous containing modified 9K medium.

Cultures were purified by serial dilution in 15 test tubes having 10^{-1} to 10^{-15} dilution of inoculum. After 2 weeks of incubation, tubes having dilutions from 10^{-1} to 10^{-8} showed rust coloration of medium because of microbial growth and ferrous oxidation. Individual cultures from highest dilution tube (10^{-8}) were subcultured in fresh 9K medium with 1% ferrous sulphate and their iron oxidation rate was determined, which is given in table 4.1.

The isolates gave iron oxidation rate in range of 142 to 570 $mg.L^{-1}.h^{-1}$ in 1% ferrous sulphate containing medium. Highest iron oxidation was observed with SRD 5, which was 570 $mg.L^{-1}.h^{-1}$. When all the ferrous

Table 4.1: Iron and sulphur oxidation rates of *A. ferrooxidans* isolates obtained from different mining sites

No.	Isolate	Mine	State	IOR ($mg.L^{-1}.h^{-1}$)	SOR ($mg.L^{-1}.h^{-1}$)
1	SRD 5	Ambamata Polymetallic mine	Gujarat	570	19.3
2	Culture E'			228	65.8
3	HGM 17	Hutti Gold Mine	Karnataka	412	15.3
4	HGM 26			292	25.8
5	HGM 30			298	16.5
6	HGM 38			142	21.1
7	2MC	Malanjkhand copper mine	Madhyapradesh	279	28.5
8	Lig Tf	Neyveli lignite mine	Jharkhand	336	19.7

grown cultures were transferred in medium containing 1% sulphur as sole energy source, highest sulphur oxidation rate was shown by isolate E', which was 65.8 $mg.L^{-1}.h^{-1}$. This was followed by isolates 2MC and HGM 26, which were 28.5 and 25.8 $mg.L^{-1}.h^{-1}$ respectively. Lowest sulphur oxidation rate was given by isolate HGM 17, which was 15.3 $mg.L^{-1}.h^{-1}$.

Isolate SRD 5 was isolated from column reactor used for leaching of polymetallic ores containing copper, lead and zinc predominantly, and hence it was exposed to these metals present in ore. Moreover it gave highest iron oxidation rate. All these factors made this isolate more suitable for leaching. To develop this culture for leaching, it was exposed to higher concentrations of ferrous sulphate. After adaptation in 1%, 2% and 5% ferrous sulphate, SRD 5 was exposed to 10% ferrous sulphate containing 9K medium.

Eventhough isolate SRD 5 gave 570 $mg.l^{-1}.h^{-1}$ IOR in presence of 1% ferrous sulphate, during the first cycle of oxidation of 10% ferrous

Table 4.2 : IOR study of culture SRD 5 on 10% $FeSO_4$

No of cycles*	$FeSO_4$ conc. (%)	Total Iron (%)	Total Iron (mM)	Overall IOR ($mg.L^{-1}.h^{-1}$)	Reaction time (h)	Highest IOR ($mg.L^{-1}.h^{-1}$)
1	10	10	719	93	237	116
2	10	13.2	475	145	166	240
3	10	16.6	597	187	95	319
4	10	18.4	662	438	42.5	460
7	10	19.8	712	419	47	471
10	10	19.975	718.5	482	48	628
11	10	19.99	719	532	43	746

* In each cycle, fresh 9K medium flask with 50% inoculum and 10% FeSO4 was taken.

sulphate, an IOR of only 93 $mg.L^{-1}.h^{-1}$ was obtained (Table 4.2) and biooxidation completed in 237 h (~10 days). During the 237 h of incubation, highest iron oxidation was observed between 24 to 48 h and it was 116 $mg.L^{-1}.h^{-1}$. Subsequent transfer of isolate SRD 5 in fresh 9K medium containing 10% ferrous iron resulted in decrease in time required for >99% iron oxidation. As compared to 237 h required in 1^{st} cycle, in 11^{th} cycle of subculturing almost complete oxidation was achieved in 43 h only.

During this time, the highest IOR of each cycle increased from 116 $mg.L^{-1}.h^{-1}$ to 319, 460 and 746 $mg.L^{-1}.h^{-1}$ at 3^{rd}, 4^{th} and 11^{th} cycles respectively (Table 4.2). Since this sub-culturing was carried out to increase rate of iron oxidation, after completion of 1^{st} cycle of 10% iron oxidation, 32 ml inoculum from total 100 ml reaction mixture was transferred as inoculum into 68 ml fresh 9K medium. In this way, 32% ferric from previous broth was also transferred to new flask in which 10%

ferrous sulphate was added for oxidation. Hence total iron content of medium (cumulative of ferrous and ferric) reached to 13.2%.

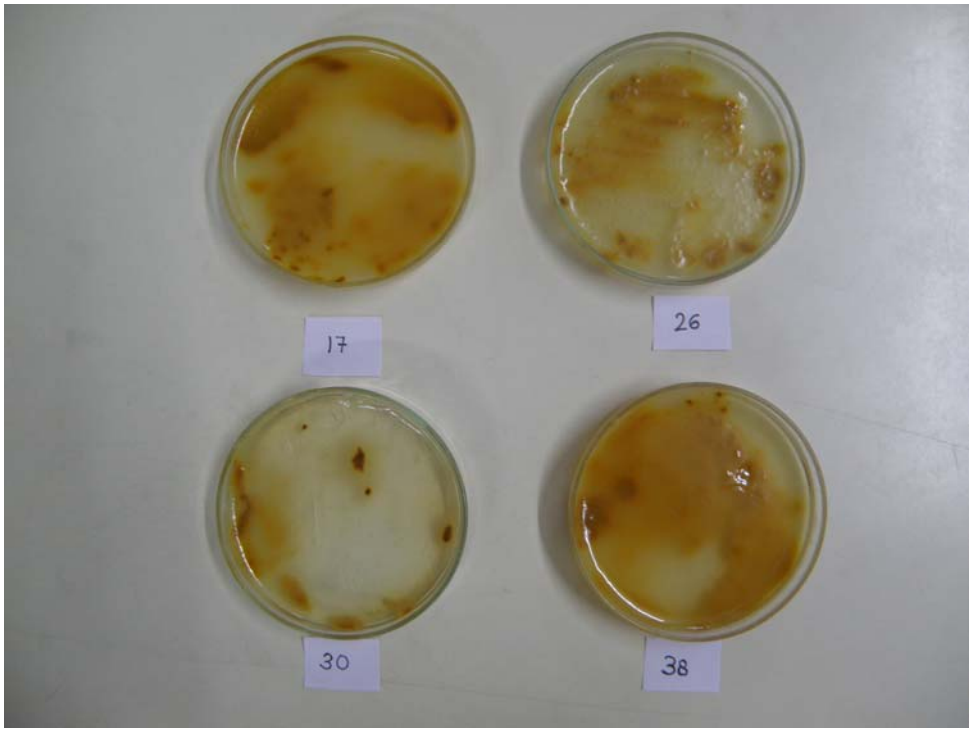
After 2nd cycle, in all further batches 50% reaction mixture was used as inoculum. From 2nd cycle to 11th cycle the total iron concentration increased from 13.2 % to 19.99%, which amounted to 475 mM to 719 mM. Available literature shows that ferric sulphate concentration more than 10 $g.L^{-1}$ inhibits the ferrous biooxidation and growth of iron oxidizers.

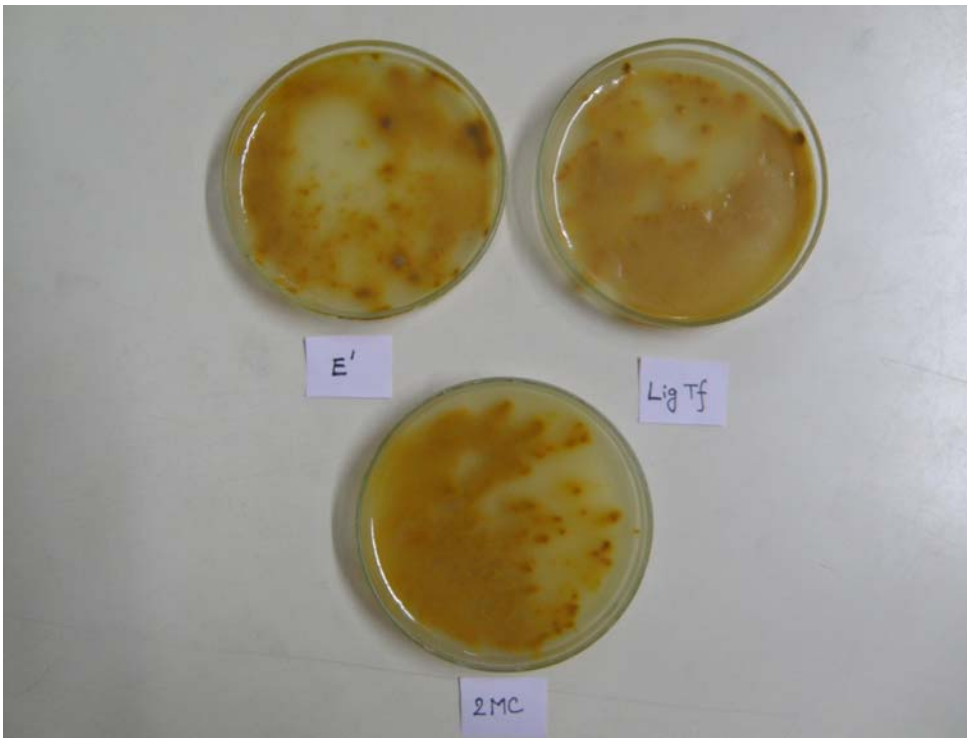
In first cycle, the isolate was exposed to 10% ferrous sulphate for first time. It took 237 h to complete the reaction, but on second cycle the isolate had adapted for oxidation of 10% ferrous sulphate, hence >99% oxidation was completed in only 166 h and in 3rd cycle the reaction took only 95 h.The reaction time for oxidation decreased in each cycle and in 11th cycle, it took only 43 h for almost complete oxidation.

The isolates were grown on DBJ solid medium to study the colony morphology of each isolate (Johnson *et al.* 1987; Johnson and McGinness, 1991). On solid medium (using agarose) with low concentration of ferrous sulphate, microscopic colonies were formed giving amber zone in the medium around them. All the isolates gave small microscopic colonies on DBJ agar plates on 12th day. The medium surrounding microscopic colonies became rust coloured by oxidation of ferrous iron and till 14th day small irregular colonies became visible with ferric deposition on them. Photograph 4.1 shows the colonies obtained on solid medium on 14th day.

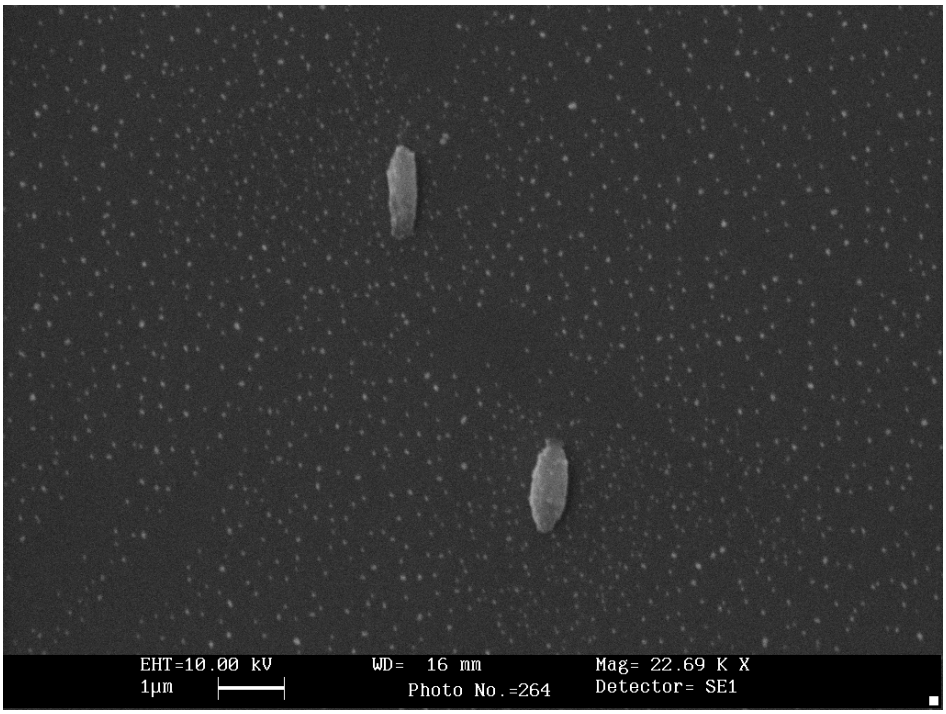
The morphology of each culture was ascertained by Scanning Electron Microscopy as shown in photograph 4.2, 4.3 and 4.4. By SEM, Culture SRD 5 appeared as short plump rods having dimension of 1.3 μm X 0.5 μm at 22690 X magnification. Isolate HGM 26 appeared slender as compared to SRD 5 at 26430 X magnification. The size of isolate HGM 26 was 1.2 μm X 0.4 μm as observed in photograph 4.3. Shrinkage of cellular cytoplasm was also observed by prolonged exposure of cells.

Photograph 4.1: Colonies of iron oxidizing isolates on DBJ medium.

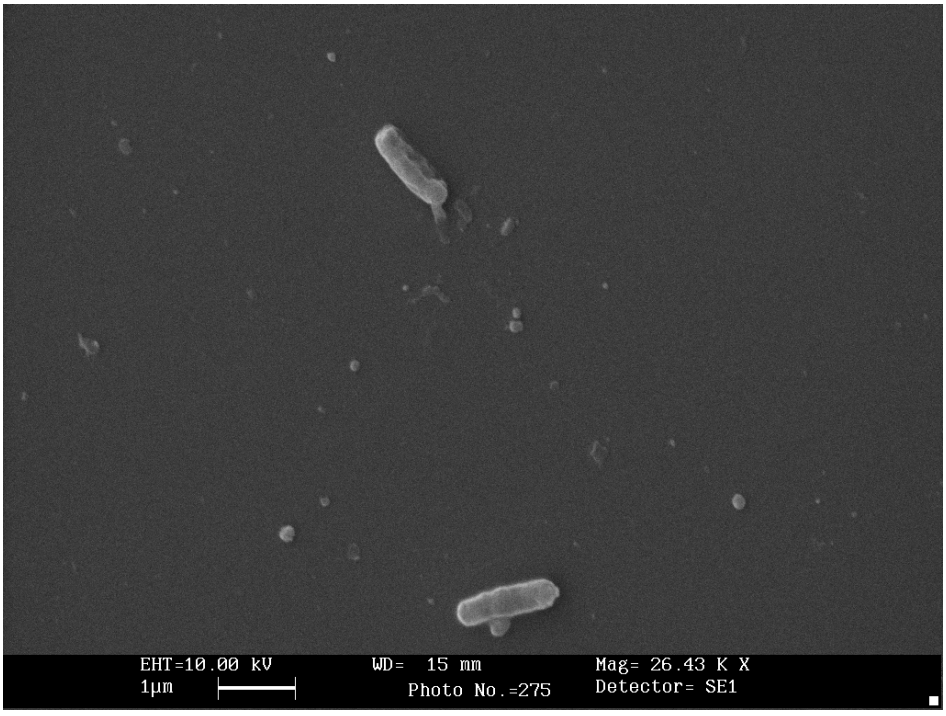




Photograph 4.2: SEM image of SRD 5 at 22690 X magnification



Photograph 4.3: SEM image of HGM 26 at 26430 X magnification



Photograph 4.4: SEM image of HGM 38 at 50000 X magnification

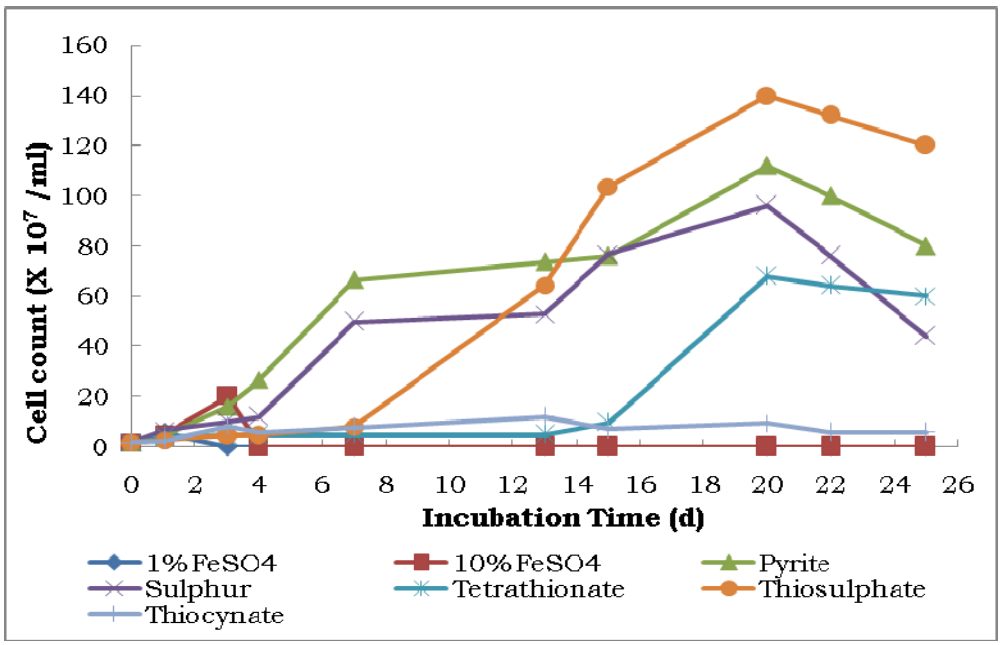
Morphology of HGM 38 was very different as compared to other isolates, since cells of HGM 38 appeared as curved rods having approximately 1.5 μm X 0.3 μm size.

4.3.2 GROWTH IN PRESENCE OF DIFFERENT ENERGY SOURCES

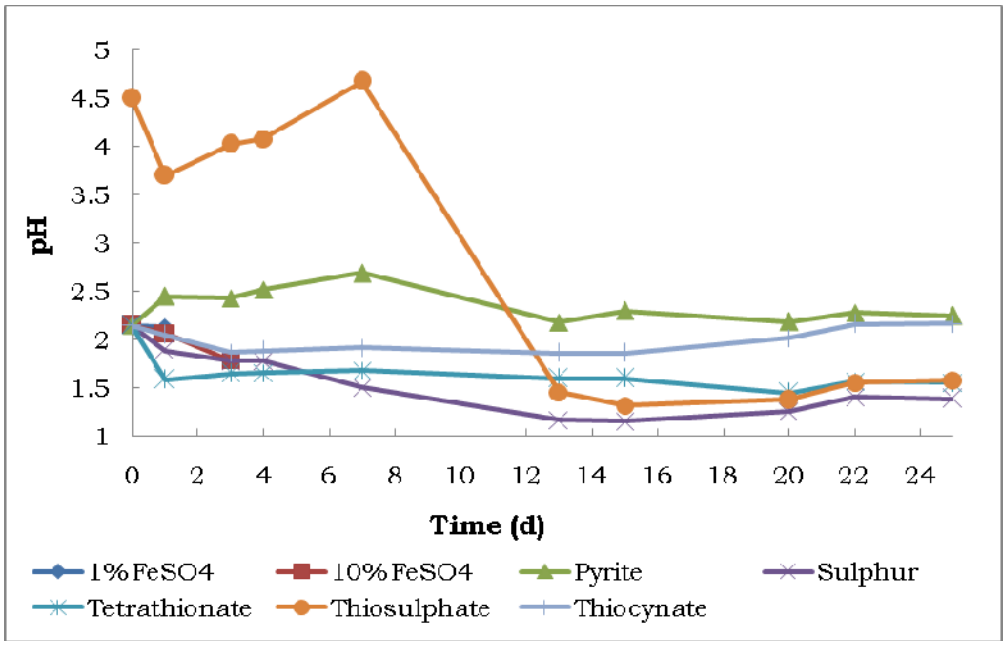
4.3.2.1 SRD 5

When 3 isolates SRD 5, HGM 26 and HGM 38 were compared for their response to conventional energy sources like pyrite, sulphur, thiosulphate, thiocyanate and tetrathionate, maximum growth of SRD 5 was supported by thiosulphate, which gave 87.5 fold increase in cell load in 20 days as shown in graph 4.1. During initial 7 days there was nominal increase in cell count. It could be due to the adaptation time required by the iron grown cells to adapt to thiosulphate oxidation. As shown in graph 4.2, during this time the medium pH showed slight increase from 4.5 to 4.67, indicating very little utilization of thiosulphate. After 7^{th} day the pH fell from 4.67 an on 13^{th} day it was 1.46, by which time oxidation had started.

Graph 4.1 : Cell growth of SRD 5 on conventional energy sources.



Graph 4.2 : pH change during growth of SRD 5 on conventional energy sources.



Using pyrite as energy source, the cell count of SRD 5 increased vigorously from 1.6 X 10^7 cells/ml on 0 day to 4.2 X 10^7 cells/ml (2.6 fold increase) after 24 h and 1.6 X 10^8 cells/ml (10 fold increase) after 72 h. The gradual increase continued till 20 days, by this time 70 fold increase in cell count was observed. After that, decrease in cell count took place. As pyrite was oxidized, it resulted in increase of pH for initial 7 days which reached upto 2.69 pH on 7th day. This increase was observed since pyrite was not acid stabilized before use and resulted in alkali generation because of gangue material present within sample. After 7 days alkalinity due to gangue material was neutralized and gradual decrease in pH was observed.

When sulphur was used as energy source, it gave nominal rise in cell count till 4th day. During this time only 7.5 fold increase in cell growth was observed. This slow rise in cell count is expected, since the culture was grown on ferrous iron and has no previous exposure to sulphur. Hence cells require some time to adapt on sulphur as energy source and switching their energy generation mechanism from ferrous to sulphur. Suzuki *et al.* (1990) reported that all the *T. ferrooxidans* strains studied had the ability to produce cells with Fe^{2+} and S^0 oxidation and Fe^{3+} reduction activities, but their levels were influenced by growth substrates and strain ability to oxidize Fe^{2+}.

After 4th day, the cell count increased from 1.2 X 10^7 cells/ml to 5 X 10^7 cells/ml from 4th day to 7th day and reached upto 10 X 10^7 cells/ml on 20th day. This proved to be third best energy source for SRD 5 after thiosulphate and pyrite. However, when SRD5 grew on sulphur, it showed continuous decrease in pH during period of adaptation and cell growth. The medium pH decreased from 2.15 on inoculation day to 1.39 on 25th day.

In presence of tetrathionate, the cell load of SRD5 increased only 3 fold from inoculation day to 13th day. After 13th day, rapid increase in cell growth was observed. The cell count increased 2 fold from 13th day to

15th day and reached 14 fold on 20th day. This made tetrathionate an important energy source for SRD5 after thiosulphate, pyrite and sulphur. During the course of growth, the pH decreased to 1.6 within 24 h and remained stable thereafter. The rapid rise in cell count after 13th day also did not result in significant pH change.

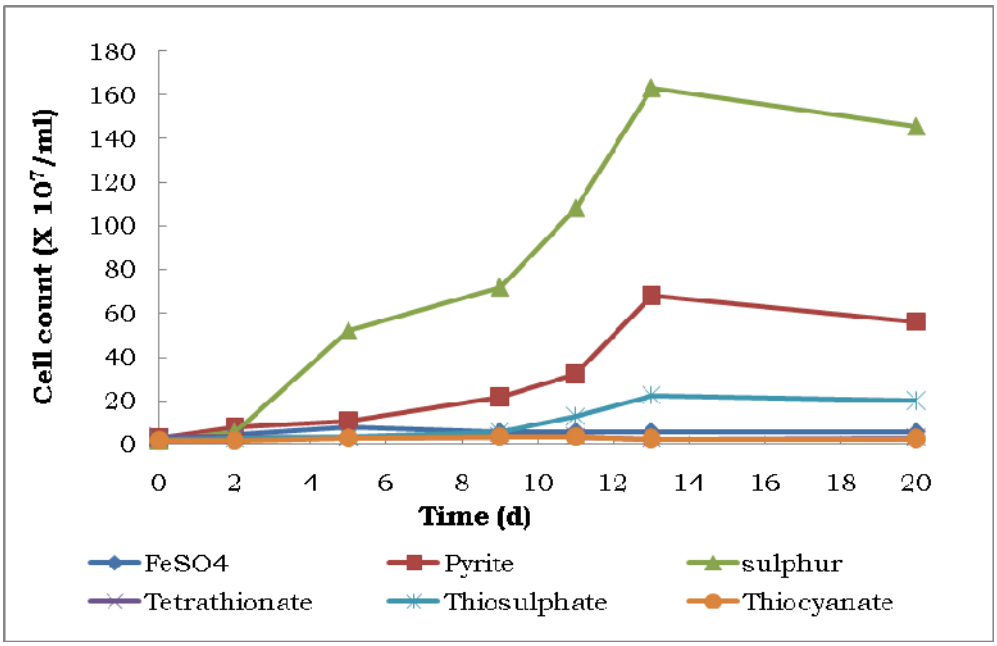
Thiocynate in medium served as poor energy source for SRD 5 and cell count increased only 7.5 fold from 1.6 X 10^7 cells/ml to 1.2 X 10^8 cells/ml in 13 days. Along with gradual increase in cell count, medium pH decreased from 2.15 to 1.86 in 15 days.

4.3.2.2 HGM 26

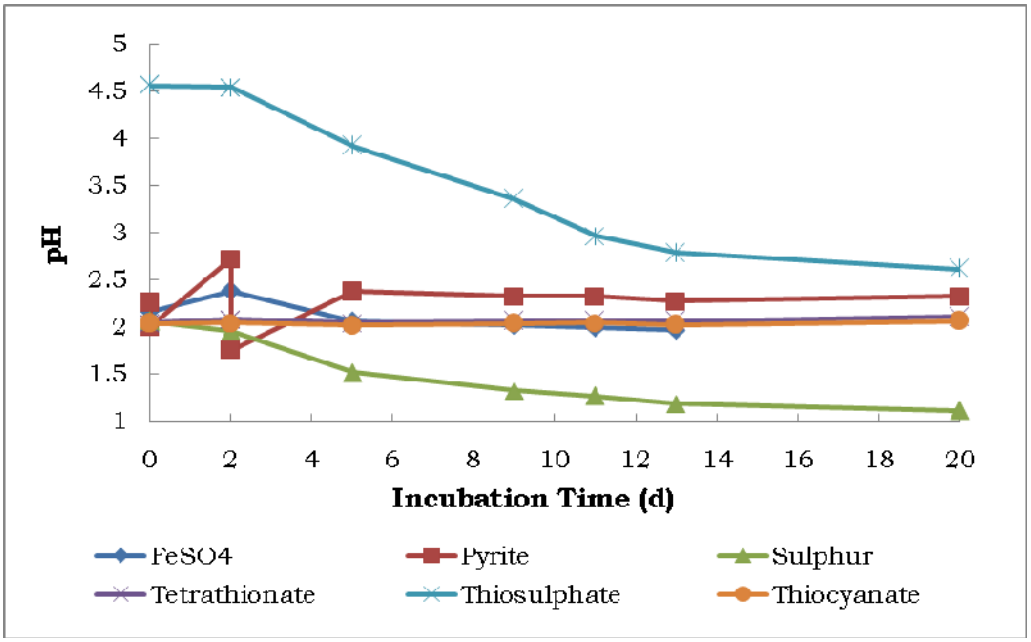
HGM 26 showed highest growth when sulphur was used as energy source (Graph 4.3). Similar to the other two isolates SRD 5 and HGM 38, growth of HGM 26 was also slow during 48 h, whereby its cell count increased from 1.6 X 10^7 cells/ml to 5.6 X 10^7 cells/ml (3.5 fold increase). This may be due to adaptation of ferrous sulphate grown cells to sulphur as sole energy source. During this time, little decrease in medium pH was observed, which was from 2.06 on 0 day to 1.95 on 2nd day (Graph 4.4). After 48 h, the cell count increased exponentially to 5.2 X 10^8 cell/ml, 1.1 X 10^9 cells/ml and reached to 1.6 X 10^9 cells/ml on 5th day, 11th day and 13th day respectively, after which cell count decreased continuously till 20 days. Along with increase in cell count, medium pH decreased continuously till 20 days of experiment from 1.5 pH on 5th day to 1.26, 1.18 and 1.11 pH on 11th, 13th and 20th days respectively.

HGM 26 grew slowly on pyrite as compared to isolate SRD 5. Maximum increase in growth of HGM 26 was 42.5 fold after 13 days (Graph 4.3). As against this, 46 fold increase was observed in SRD5 during same time period. The cell load of SRD 5 still increased and reached upto 70 fold in 20 days. When HGM 26 was inoculated in medium containing 1% pyrite, it did not show good growth till 7-8 days.

Graph 4.3 : Cell growth of HGM 26 on conventional energy sources.



Graph 4.4: pH change during growth of HGM 26 on conventional energy sources.



During this time only 7.7 fold increase in cell load was observed. The medium pH increased from 2.25 to 2.72 in 2 days, which was decreased to 1.75 by addition of sterile 10% H_2SO_4 on the same day. The pH again increased to 2.37 on 5th day but remained stable after that.

In presence of thiosulfate as sole energy source, the cell count did not increase till 5 days. Simultaneously there was gradual decrease in pH from 4.56 on 0 day to 3.92 on 5th day (Graph 4.3, 4.4). This pH was unfavorable for growth of HGM 26. On 9th day 3.35 pH was reached. At this pH, cell growth started and it showed 1.6 fold growth on 5th day and reached to 2.8 fold on 9th day. After 9th day the decrease in pH was very fast and it decreased from 3.35 to 2.78 in 20 days. Simultaneously the cell count increased to 11.2 fold on 13th day, but after 13th day cell count did not increase further.

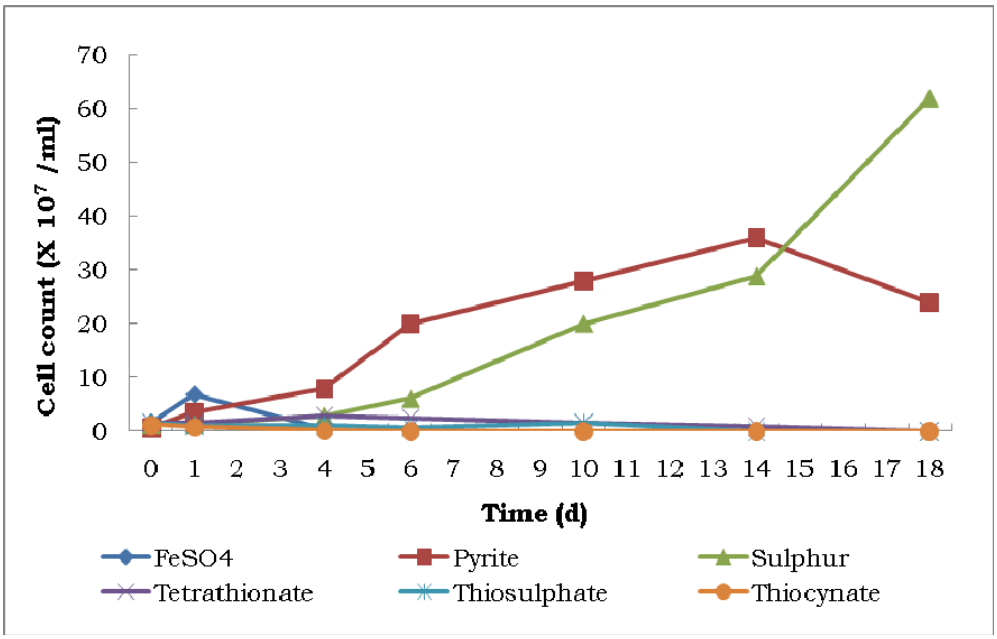
4.3.2.3 HGM 38

Sulphur proved to be the best energy source for isolate HGM 38. In presence of sulphur, there was only 12 fold increase in growth in initial 6 days (Graph 4.5). This delay is expected as organism adapt to a new energy source, if they have not been exposed to it before. After adaptation on sulphur as energy source, cell load gradually increased from 6 X 10^7 cells/ml on 6th day to 2.88 X 10^8 cells/ml (18 fold) and 6.2 X 10^8 cells/ml (39 fold) on 14th and 18th day respectively.

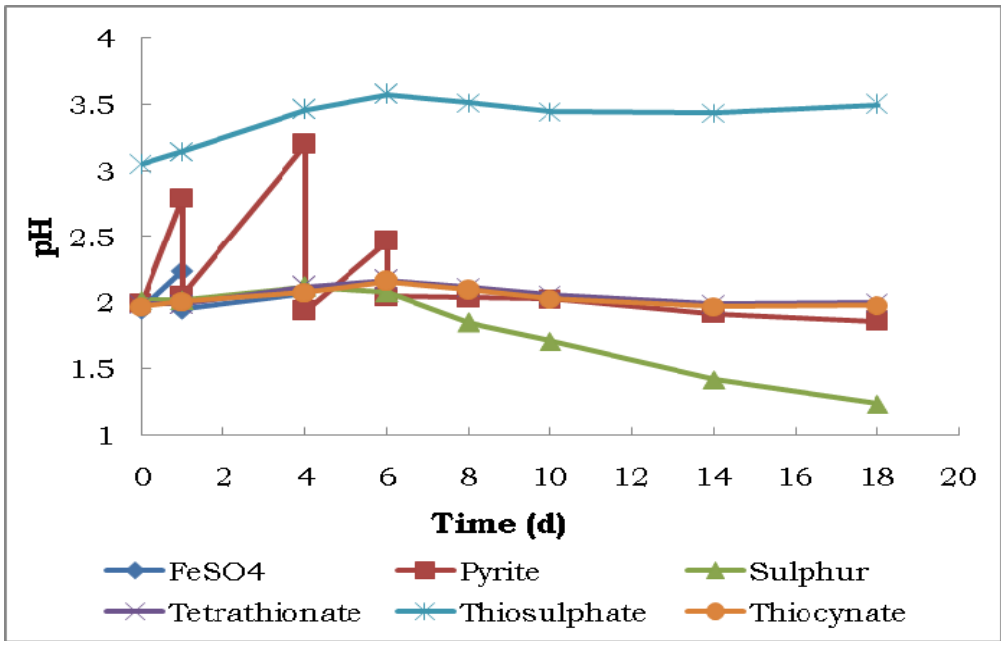
The same pattern was observed in its pH profile also (Graph 4.6). pH in sulphur containing medium remain somewhat unchanged till 6 days, after which rapid decline in pH was observed and it reached to as low as 1.42 and 1.24 in 14 and 18 days respectively.

When pyrite was used as energy source, there was slow increase in cell count for initial 4 days. This slow rise in cell count could be attributed to the alkalinity generated in the medium during initial stages of pyrite biooxidation. This alkalinity is due to gangue material present within pyrite, which has to be neutralized by addition of sterile sulfuric acid at

Graph 4.5: Cell growth of HGM 38 on conventional energy sources.



Graph 4.6: pH change during growth of HGM 38 on conventional energy sources.



regular intervals. After 24 h of growth, pH rose from 2 to 2.8, which was brought down to 2 by acid addition. This pH again increased to 3.2 on 4th day which was brought down upto 2 the same day. Again when checked on 6th day, it had increased to 2.47 and it was brought down to 2. As pH increase was controlled within 2.5, it became suitable for growth of the organism and rapid growth was observed after that. After 6 days, pH started decreasing as alkalinity of pyrite was reduced and the pH on 14th and 18th day was 1.92 and 1.86 respectively. In 14 days of growth, pyrite gave the highest growth of HGM 38, among all the energy sources used, viz. sulphur, pyrite, tetrathionate, thiosulphate and thiocynate. On 14th day it gave 22.5 fold increase in cell count as compared to 0 day. After 14 days the cell count started decreasing.

When HGM 38 was grown in presence of thiosulphate, cells did not show any growth and similarly medium pH also did not decrease till end of experiment indicating that HGM 38 was not able to use thiosulphate. However the inhibition of thiosulphate utilization can be due to the presence of unoxidized ferrous in the inoculum. Das *et al.* (1993) has also reported that thiosulphate and tetrathionate oxidation activity of *T. ferrooxidans* were found to be absent in iron grown cells and it remained absent till the oxidation of ferrous iron was complete. The cells harvested only in later period acquired the thiosulphate and tetrathionate oxidation activity (Das *et al.*, 1993).

None of the studied organism could use thiocyanate or tetrathionate as energy source since no increase in cell load was observed and no change in pH was noticed during entire experiment.

4.3.3 GROWTH ON METAL SULPHIDES

Soluble metal ions are frequently present in fairly high concentrations in highly acidic environments. Metal ions which exist in more than 1 oxidation state and which have redox potentials that are more negative than the O_2/H_2O redox couple, have the potential to serve as electron

donor for acidophilic bacteria (Rawlings 2005). Brierley (1978) reported the growth of *A. ferrooxidans* using energy from copper sulphide.

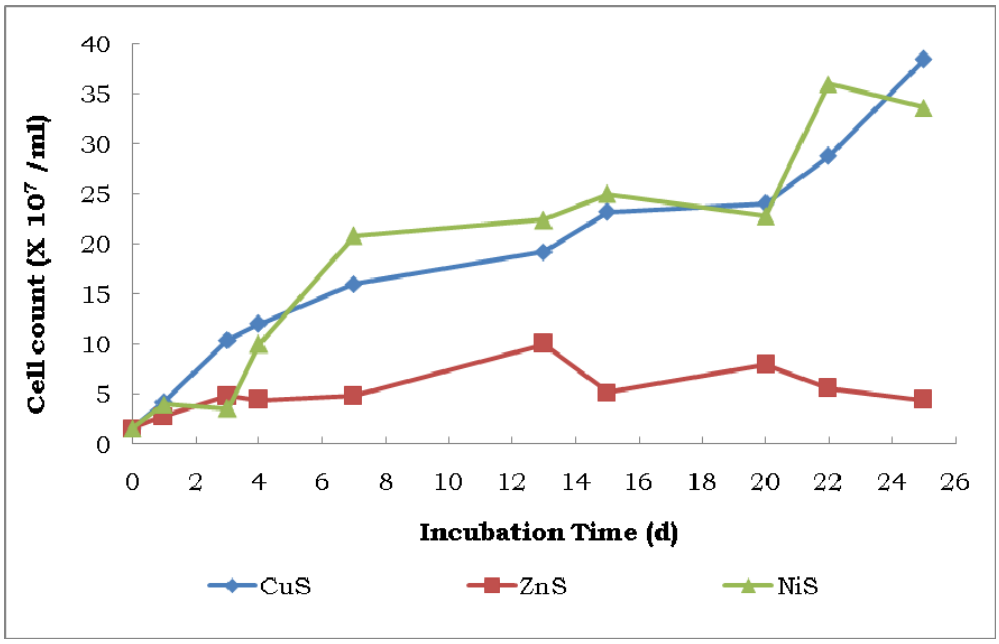
4.3.3.1 SRD 5: Copper sulphide (CuS), nickel sulphide (NiS) and zinc sulphide (ZnS) were tested as energy sources. Presence of copper sulphide in medium as a sole energy source supported growth of SRD5 and cell count increased gradually from 1.6 X 10^7 cells/ml to 3.84 X 10^8 cells/ml during 25 days of experiment (Graph 4.7). With increase in cell count, pH also decreased from 2.15 to 2.0 in 13 days and then increased and reached to 2.32 (Graph 4.8). DiSpirito *et al.* (1982) has reported *A. ferrooxidans* like bacterium which directly oxidized Cu^+ to Cu^{2+} and U^{4+} to U^{6+} under aerobic conditions and these oxidation reactions were coupled to CO_2 fixation.

As copper sulphide, nickel sulphide also supported growth of SRD 5 to some extent. Initially the cell count did not increase much during 3 days and only 2 fold increase was found. After 3 days the cell count increased 15.6 fold and 22.5 fold on 14^{th} and 22^{nd} day respectively. The cell growth in presence of nickel sulphide was better as compared to copper sulphide.

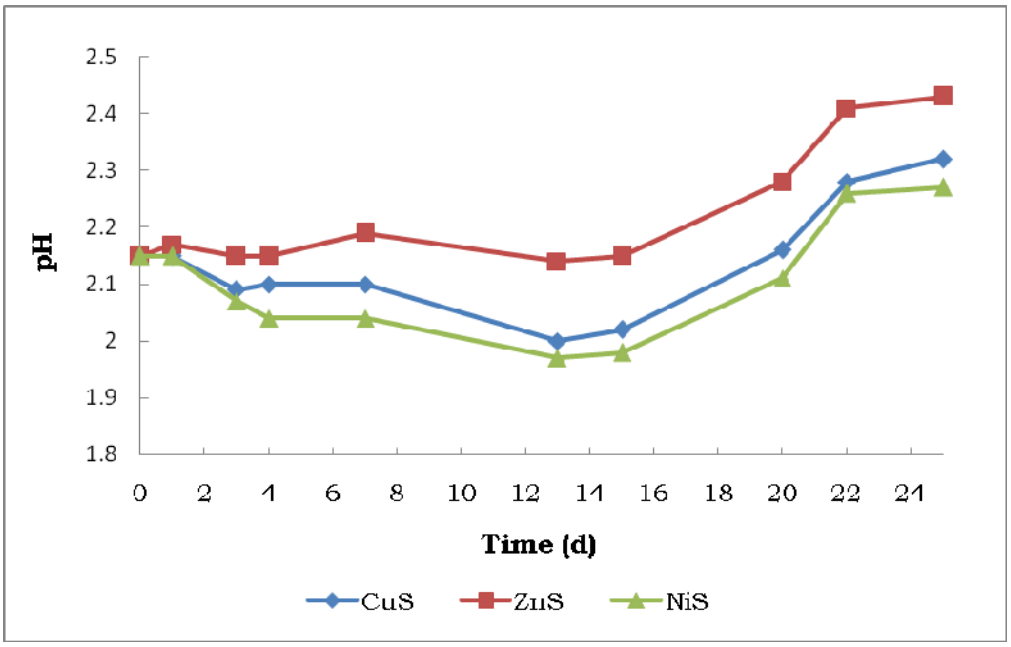
Presence of zinc sulphide as energy source gave small rise in cell load from 1.6 X 10^7 to 1 X 10^8 cells/ml (6 fold) between 0 day and 13^{th} day. Here, pH of medium remained similar for upto 15 days after which increase in pH was observed.

4.3.3.2 HGM 26: Among 3 metal sulfides tested as energy source, copper sulfide (CuS) gave best results with HGM 26 (Graph 4.9). In presence of CuS, cell count of HGM 26 increased without any lag period from 2 X 10^7 cells/ml to 3.36 X 10^8 cells/ml in 20 days of experiment. In presence of CuS, medium pH increased slightly from 1.97 to 2.06 on 2^{nd} day. After that pH remained stable at 2 till the end of experiment (Graph 4.10).

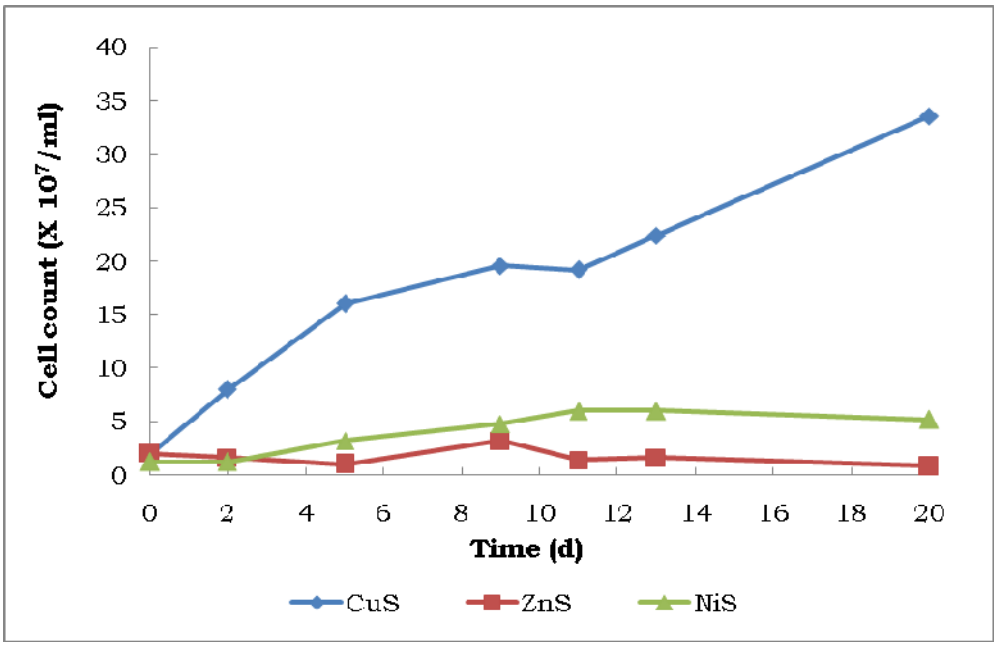
Graph 4.7: Cell growth of SRD 5 on metal sulphides.



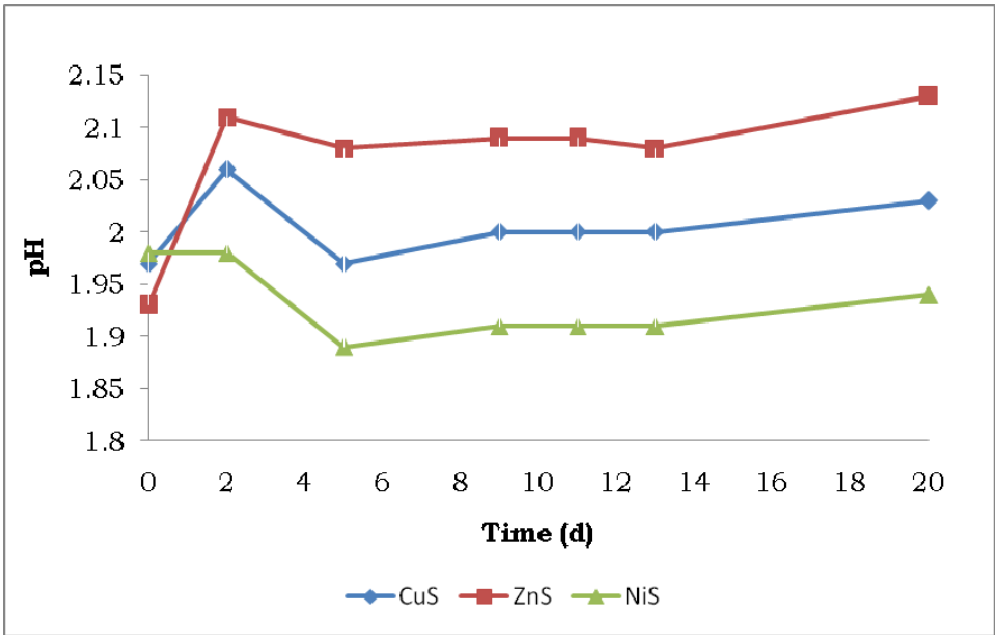
Graph 4.8: pH change during growth of SRD 5 on metal sulphides.



Graph 4.9: Cell growth of HGM 26 on metal sulphides.



Graph 4.10 : pH change during growth of HGM 26 on metal sulphides.

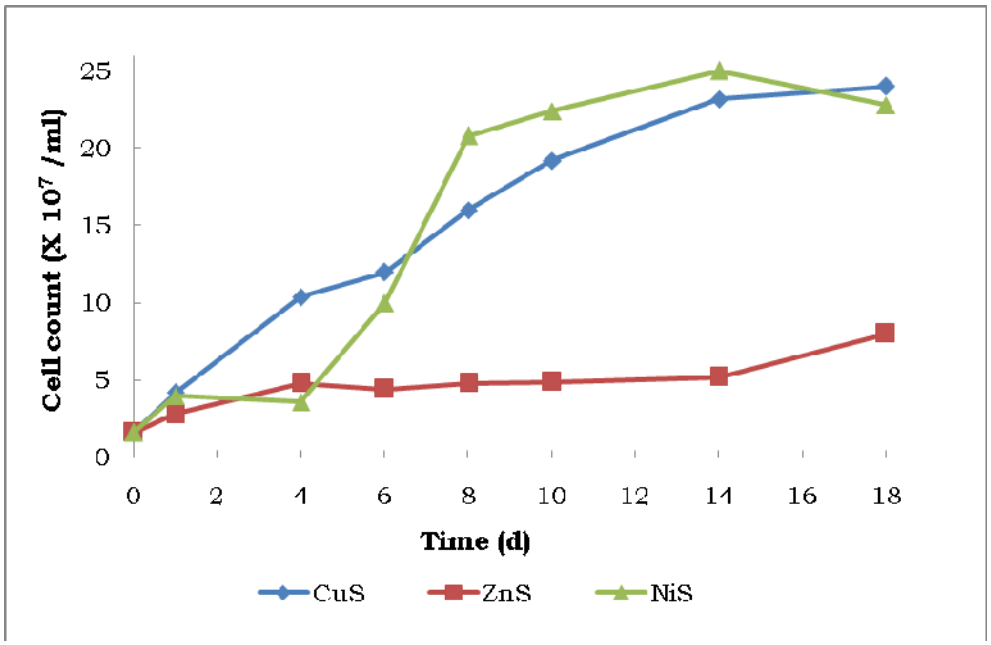


In case of NiS as energy source, the cell count showed 5 fold increase from 1.2 X 10^7 cells/ml to 6 X 10^7 cells/ml in 13 days. Initially from 0 day to 5^{th} day, the medium pH decreased slightly from 1.98 to 1.89, which then remained stable for rest of the experiment.

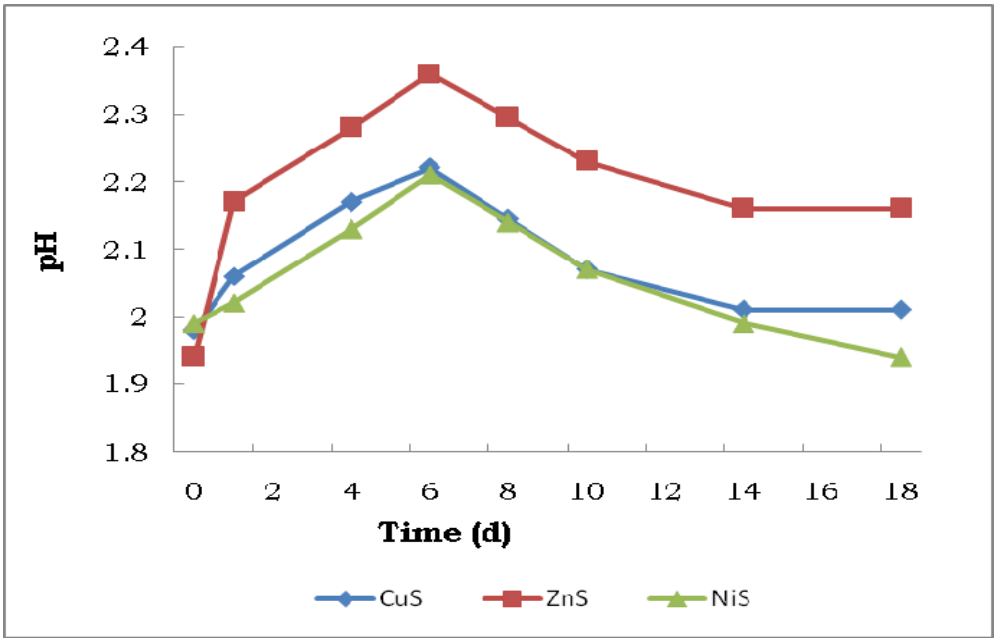
When zinc sulfide was used as sole energy source, the medium pH increased from 1.93 to 2.11 in initial 2 days of incubation, which then remained stable at 2.08 till 13 days. In presence of ZnS, the cell count did not increase from 2 X 10^7 cells/ml on day of incubation and a continual decrease of 1.6 X 10^7, 1.4 X 10^7 and 0.8 X 10^7 was noted on 2^{nd}, 11^{th} and 20^{th} day indicating inhibition due to zinc in the medium.

2.3.3.3 HGM 38: Contrary to SRD 5 and HGM 26, Nickel sulphide served as better energy source for HGM 38 among the 3 metal sulfides tested (Graph 4.11). Upto 14 days NiS gave better cell growth (15.63 fold) to HGM 38, whereas CuS gave 14 fold increase. After 14 days, however the cell count in NiS containing medium decreased, while Copper sulphate still gave exponential growth to the isolate. Zinc sulphide gave only 5 fold increase in cell count in 18 days. With all the 3 metal sulphides tested, the medium pH increased in 6 days and reached from 2.21 to 2.35, after which pH decreased till end of experiment (Graph 4.12).

Graph 4.11: Cell growth of HGM 38 on metal sulphides.



Graph 4.12: pH change during growth of HGM 38 on metal sulphides.



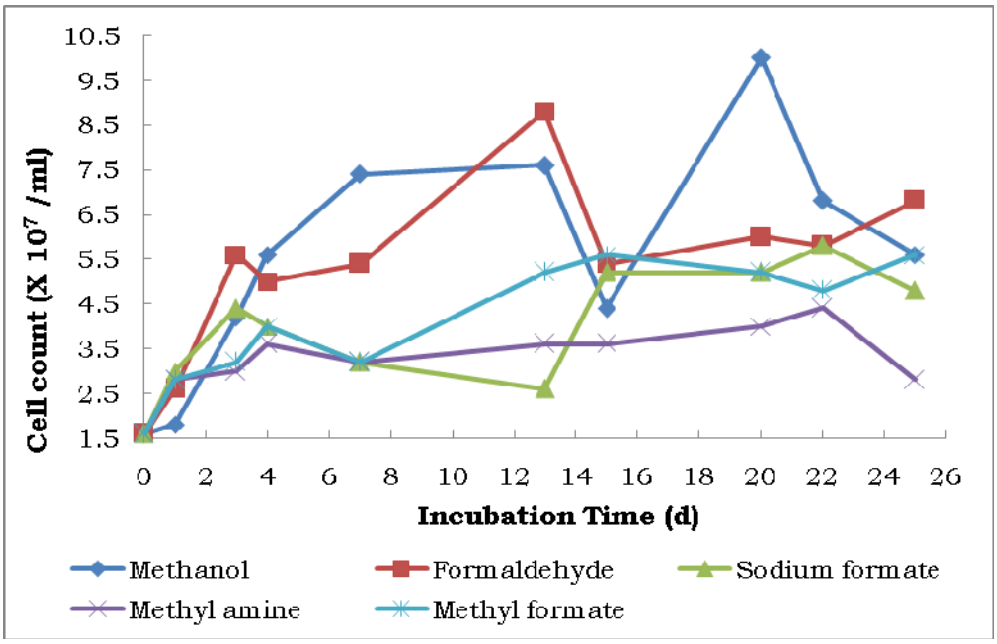
Some organic and inorganic substrates like methanol, formaldehyde sodium formate, methyl amine and methyl formate were tested for their possible use in supporting the growth of *Acidithiobacillus ferrooxidans.* Formaldehyde and methanol gave only 2 and 1.7 fold increase in cell count on HGM 26, while the same 2 chemicals gave 5.5 and 6 fold increase in SRD 5 (Graph 4.13, 4.14). Methyl formate gave 3 fold increase in SRD 5 and 1.3 fold increase in HGM 38 (Graph 4.15). Rawlings and Kusano (1994) have reported that formic acid can replace carbon dioxide as the carbon source in most of the *Acidithiobacillus ferrooxidans* strains, if the concentration of formate is kept low. Pronk *et al.* (1991) have also emphasized that *Acidithiobacillus ferrooxidans* uses formic acid as a carbon source provided that it was grown in continuous culture and the formic acid was fed-in sufficiently slowly for the concentration to remain low.

One strain of *A. ferrooxidans*, ATCC 21834 is particularly efficient in consuming formic acid. But when formic acid, acetic acid, propionic acid and butyric acid were added at a concentration of 150 mg/L, more than 94% inhibition of iron oxidation was observed in *A.ferrooxidans* and more than 85% inhibition of S^0 oxidation was observed by *A.thiooxidans.*

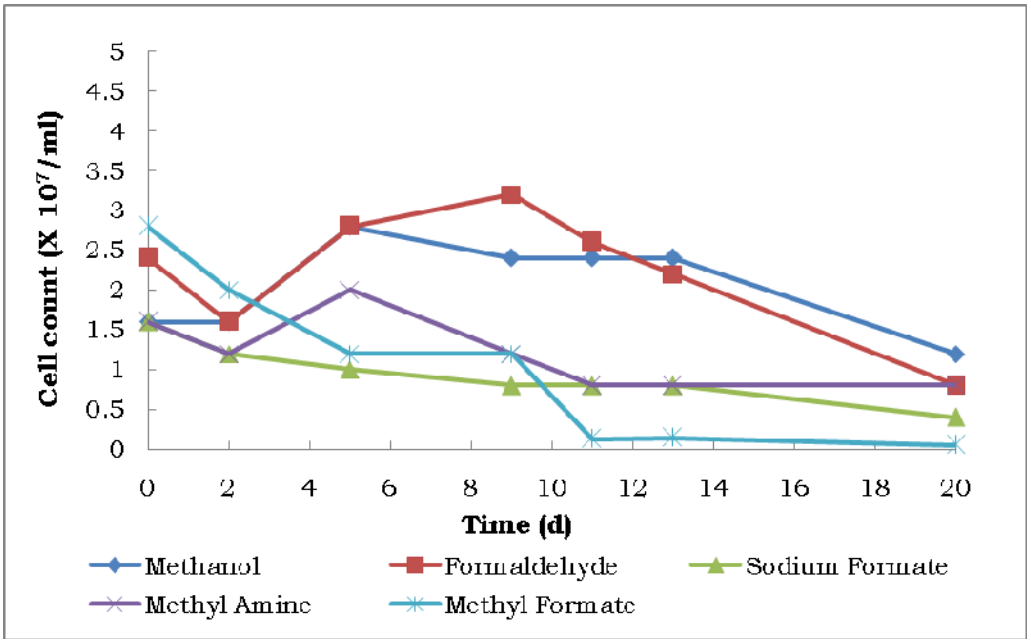
However in isolate SRD 5 and HGM 38, growth inhibition was not observed as cell count of the culture did not decrease for these 2 isolates during the course of experiment. Instead a slight increase was observed. However in HGM 26, cell count decreased continuously in presence of all the organic substances. Hence it was concluded that HGM 26 was more susceptible to studied organic chemicals as compared to SRD5 and HGM 38.

No other organic chemical gave any growth in the tested organisms. These results are summarized in Table 4.3.

Graph 4.13: Cell growth of SRD 5 on organic substrates



Graph 4.14: Cell growth of HGM 26 on organic substrates.



Graph 4.15 : Cell growth of HGM 38 on organic substrates.

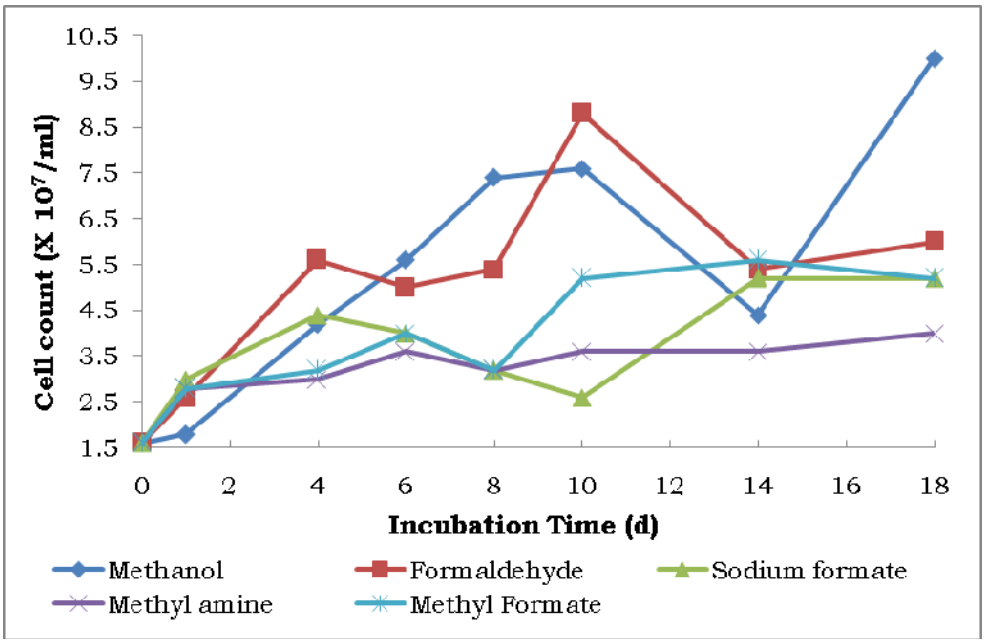


Table 4.3: Substrate utilization profile

SUBSTRATE	SRD 5	HGM 26	HGM 38
Ferrous sulphate	+	+	+
Pyrite	+	+	+
Sulphur	+	+	+
Tetrathionate	+	+	-
Thiosulphate	+	+	-
Thiocyanate	-	-	-
CuS	+	+	+
NiS	+	-	+
ZnS	-	-	-
Methanol	+	+	+
Formaldehyde	+	+	-
Sodium formate	-	-	-
Methyl amine	-	-	-
Methyl formate	+	-	+

4.3.4 EFFECT OF VARIOUS GROWTH CONDITIONS

4.3.4.1 1% thiosulphate

Three isolates SRD 5, HGM 26 and HGM 38 when exposed to various physiological conditions showed considerable difference in their response. In presence of 1% thiosulphate, isolate SRD 5 and HGM 26 showed rapid decrease in thiosulphate content and >99% thiosulphate was utilized in 7 and 10 days respectively by these 2 cultures (Graph 4.16, 4.17). However HGM 38 did not utilize thiosulphate at all.

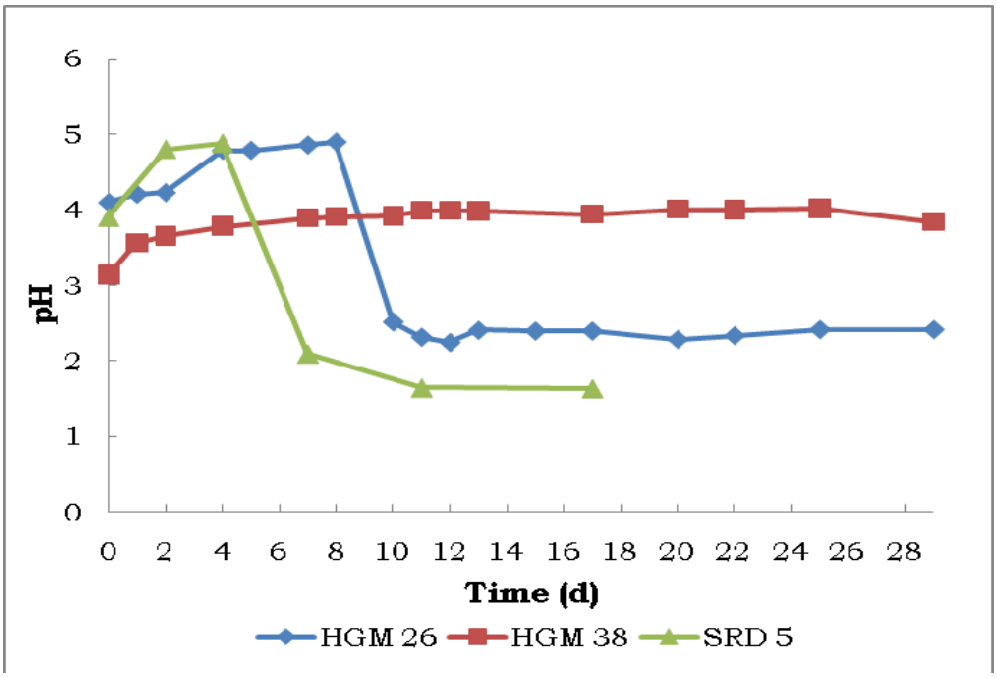
As shown in table 4.4, using thiosulphate as energy source, SRD 5 showed pH decrease from 3.71 pH on day of inoculation to 1.45 pH on 25th day. HGM 26 showed 1.58 point decrease in pH and final pH was 2.49 after 32 d. In complete contrast to these 2 isolates, HGM 38 showed increase in pH consistently till the end of experiment reaching to pH 3.84, indicating no utilization of thiosulphate.

Table 4.4 : pH change during thiosulphate and sulphur utilization

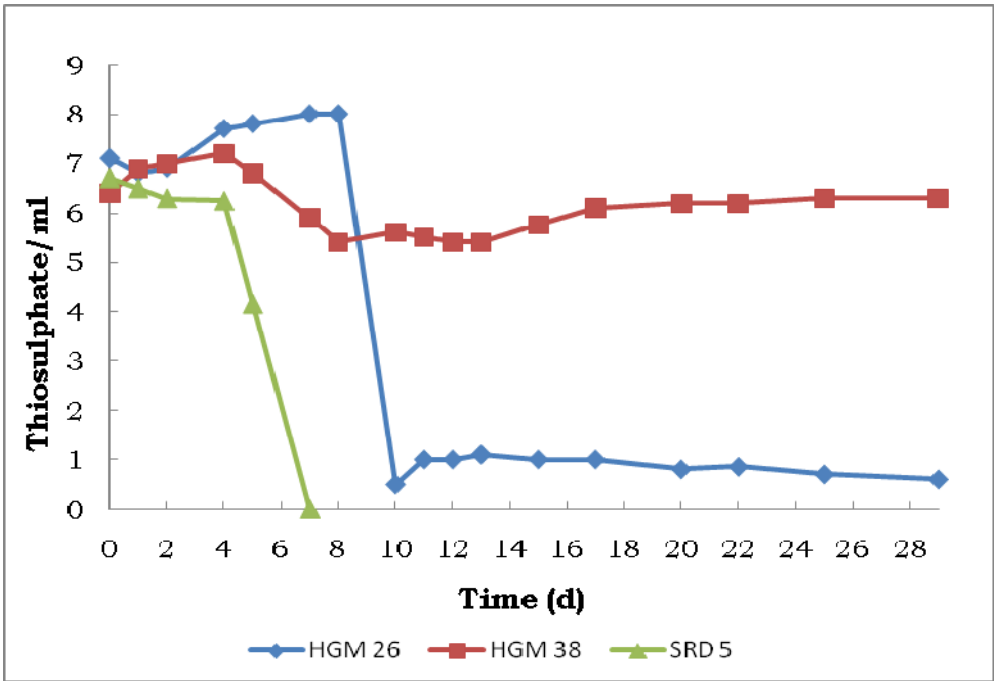
Substrate	Difference in pH in 10d			pH end point		
	SRD5	HGM 26	HGM 38	SRD5	HGM 26	HGM 38
Thiosulphate (1%)	-2.26	-1.58	+0.86	1.45	2.49	3.84
Sulphur (1%)	-2.48	-1.64	-0.54	0.83	1.21	2.31

4.3.4.2 1% sulphur : In presence of 1% sulphur as sole energy source isolate SD5 showed highest decrease of 2.48 in pH and it reached as low as 0.83 in 25 days (Graph 4.18). Next higher decrease in pH was observed in isolate HGM 26 in which pH decreased 1.64 points and a final pH of 1.21 was observed. HGM 38 showed only 0.54 decrease in 26 days and reached a lowest pH of 2.31 after 29 days (Table 4.4).

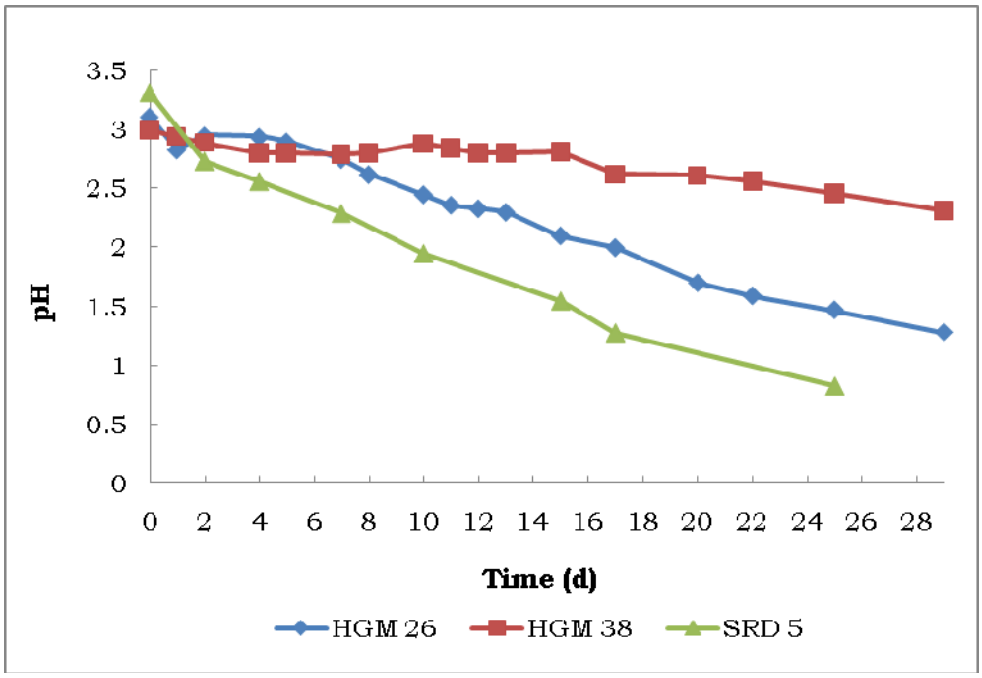
Graph 4.16: pH change during growth of 3 cultures on 1 % thiosulphate.



Graph 4.17: Utilization of 1 % thiosulphate by 3 isolates.



Graph 4.18 : pH change during growth of 3 cultures on 1 % sulphur.



4.3.4.3 6% thiosulphate: When 6% thiosulphate was used as energy source SRD5 showed only 10% utilization of thiosulphate in 10 days time. Moreover the cell count of SRD5 did not decrease till 25 days. It means SRD 5 could derive some energy from 6% thiosulphate. However HGM 26 and HGM 38 did not show any response to 6% thiosulphate as sole energy source and their cell count decreased exponentially.

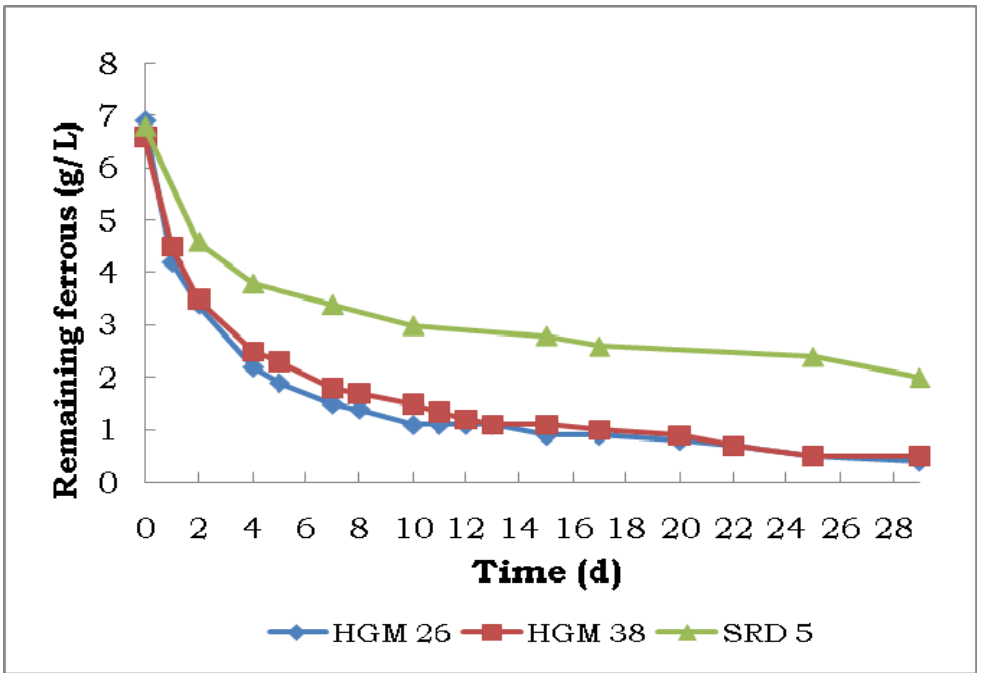
4.3.4.4 4% KH_2PO_4 : Growth of all 3 isolates was observed in presence of 4% additional PO_4 salt in 9K medium with ferrous sulphate as energy source. The effect of PO_4 was observed on iron oxidation. Harahuc *et al.* (2000) found that PO_4 concentration higher than 10mM inhibited iron oxidation and at 100mM iron oxidation was almost stopped. The effect of additional phosphate on iron oxidation is shown in graph 4.19.

In absence of any additional phosphate salt, SRD 5 carried out almost complete oxidation of 10% $FeSO_4$ and an IOR of 0.39 $g.L^{-1}.h^{-1}$ was observed. With addition of phosphate, the IOR decreased to 0.13 $g.L^{-1}.h^{-1}$ in the same duration. However 70.6% ferrous iron was oxidized in presence of 4% PO_4 (graph 4.20).

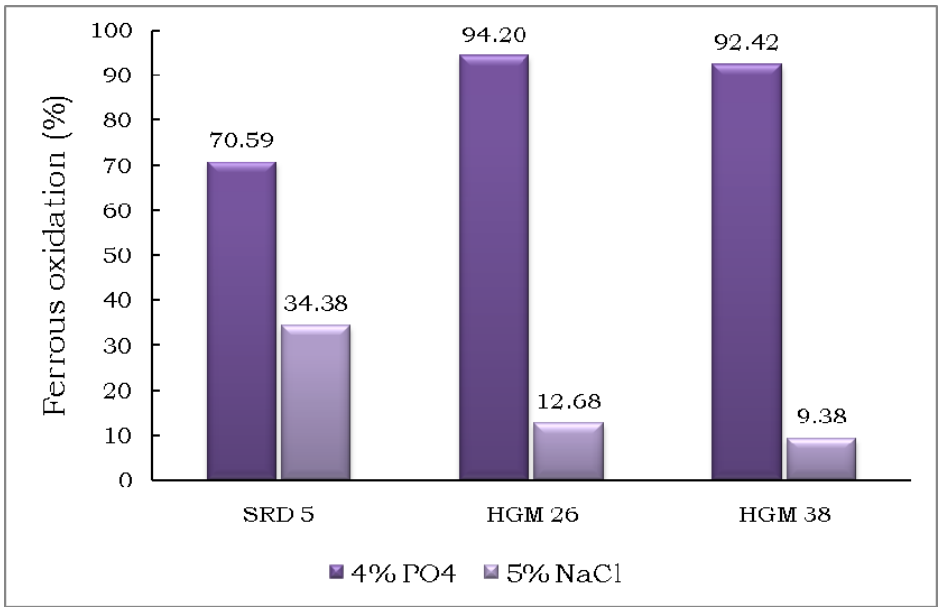
In absence of additional 4% phosphate, isolate HGM 26 oxidized 4% ferrous sulphate completely, giving an IOR of 0.096 $g.L^{-1}.h^{-1}$. Presence of phosphate in ferrous sulphate medium resulted into 62.5% decrease in IOR. Here iron content decreased gradually till 32 day and 94.2% iron was oxidized till then. In case of HGM 38, IOR in control flask was 0.15 $g.L^{-1}.h^{-1}$, which in presence of additional phosphate decreased by 66.6%. Till the end of experiment 92.4% iron had been oxidized.

Hence, the effect of 4% additional phosphate in medium was more pronounced on iron oxidation activity of isolate SRD 5, as it was able to complete only 70.6% iron oxidation (Graph 4.19 and 4.20). As against this 94.2% and 92.4% iron oxidation was completed by Isolates HGM 26 and HGM 38 respectively. Hence these 2 isolate appear to be more resistant to higher concentration of phosphate.

Graph 4.19: Effect of 4% PO_4 on iron oxidation.

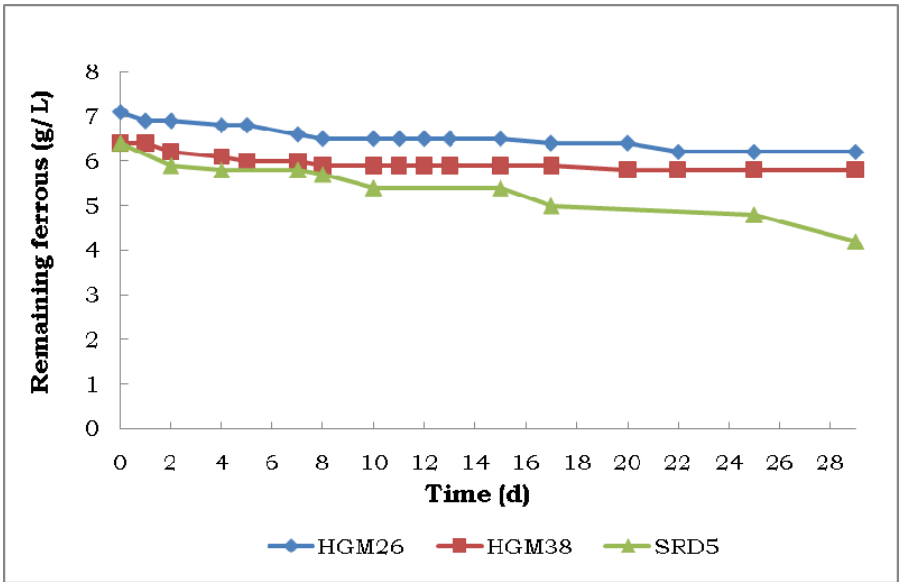


Graph 4.20: Effect of additional phosphate and NaCl on iron oxidation



4.3.4.5 5% NaCl : Presence of 5% NaCl greatly affected the organism and 65.62%, 87.32% and 90.6% inhibition in iron oxidation was observed with SRD 5, HGM 26 and HGM 38 respectively (Graph 4.20 and 4.21). None of the isolate grew in presence of KNO_3 and $NaHCO_3$ present in S8 medium (appendix II). When incubated in anaerobic medium, no isolate grew or produce gas. All the 3 isolates were inhibited on nutrient agar medium.

Graph 4.21 : Effect of 5% NaCl on iron oxidation.



4.3.5 MIXOTROPHY IN *Acidithiobacillus ferrooxidans*

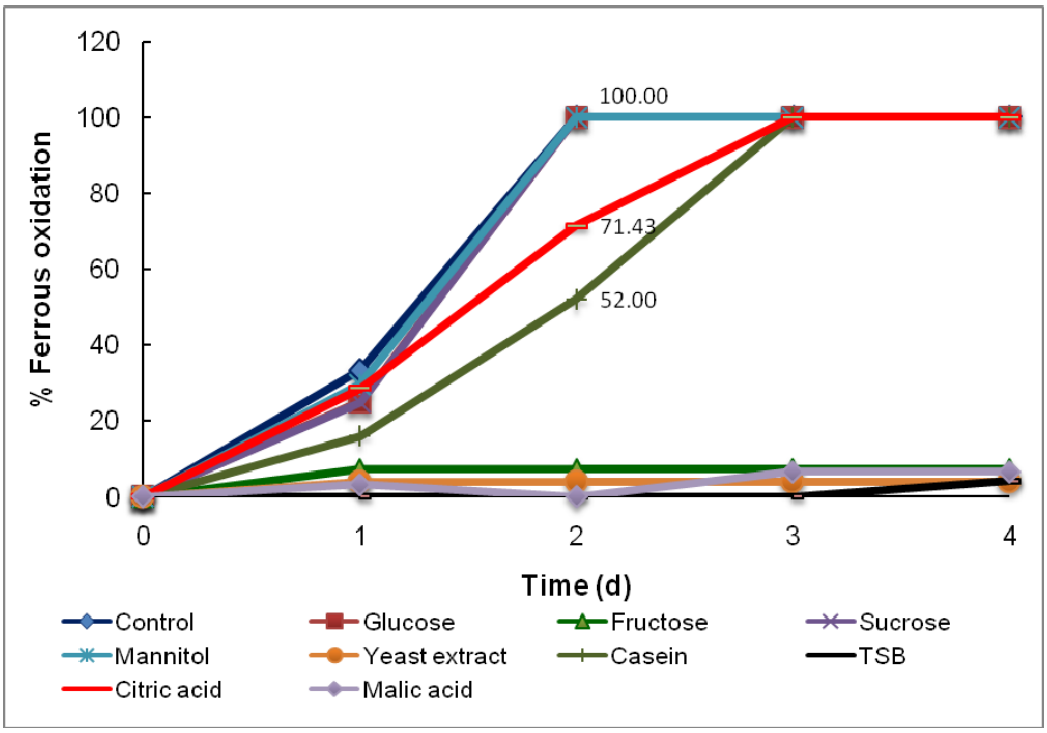
In absence of any organic supplement, isolate SRD 5 oxidized ferrous iron at the rate of 0.093 $g.l^{-1}h^{-1}$ during initial 24 h which increased to 0.14 $g.l^{-1}h^{-1}$ in 48 h (Table 4.5). However, when organic supplements such as mannitol and citric acid were added in individual ferrous sulphate containing flasks, SRD 5 gave same iron oxidation rate in initial 24 h. However in mannitol containing medium, as oxidation progressed, it supported iron oxidation and higher IOR (0.157 $g.l^{-1}h^{-1}$) was observed as compared to control flask. The presence of glucose or sucrose in the iron oxidation medium showed decreased in IOR to 0.07 $g.l^{-1}h^{-1}$ during 24 h, but reached to 0.14 $g.l^{-1}h^{-1}$ in 48 h.

Table 4.5 : Iron oxidation rate by isolate SRD 5 in presence of carbon supplement in the medium

Substrate	Iron oxidation rate ($g.L^{-1}.h^{-1}$)			
	24 h	48 h	72 h	96 h
Control	0.093	0.140	0.093	0.070
Mannitol	0.093	0.157	0.105	0.079
Glucose	0.070	0.140	0.093	0.070
Sucrose	0.070	0.140	0.093	0.070
Citric acid	0.093	0.116	0.109	0.081
Casein	0.047	0.076	0.097	0.073
Yeast extract	0.012	0.006	0.004	0.003
Malic acid	0.012	0.000	0.008	0.006
Fructose	0.000	0.000	0.000	0.000
TSB	0.000	0.000	0.000	0.003

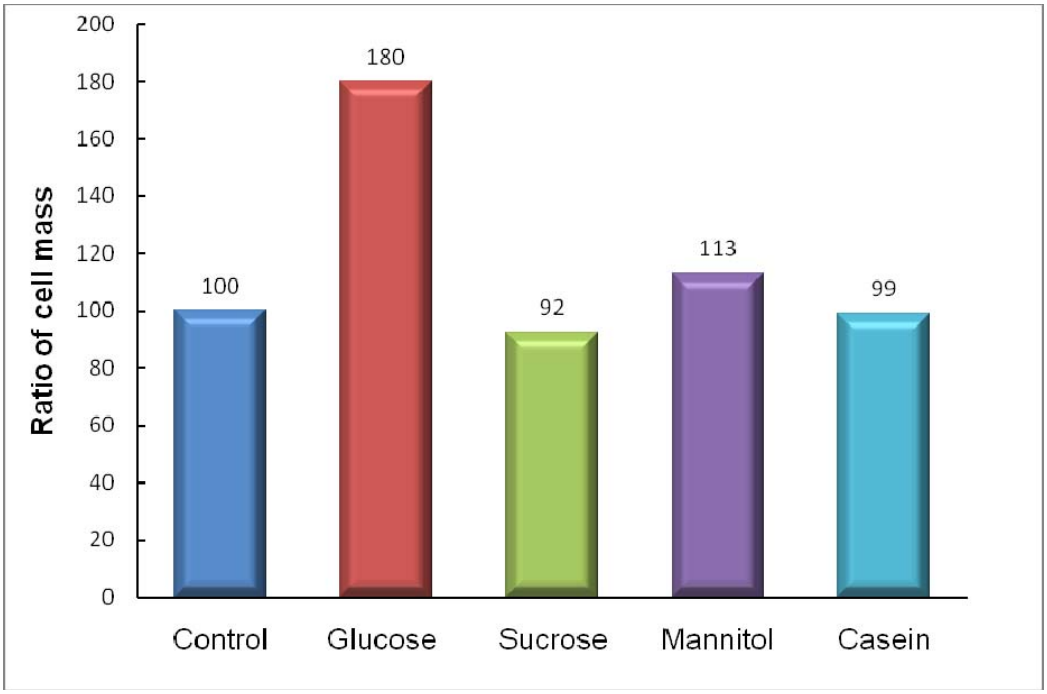
As shown in graph 4.22, more than 99% iron was oxidized by the isolate in absence as well as in presence of glucose, sucrose and mannitol in 48 h. The presence of casein and citric acid in 0.5% concentration also did not inhibited iron oxidation and >99% iron was oxidized in 72 h. The addition of fructose, yeast extract, TSB and malic acid gave only 4 to 7 % iron oxidation.

Graph 4.22 : Extent of iron oxidation by isolate SRD 5 in presence of organic carbon supplement



Even though the effect of glucose on iron oxidation was negligible and uninhibitive, it gave higher increase in cell load as compared to ferrous sulphate alone. As shown in graph 4.23, in presence of glucose in iron oxidation medium, SRD 5 showed 178% increase in cell load. Apart from glucose, addition of mannitol also gave 113% increase in cell yield as compared to ferrous sulphate alone.

Graph 4.23 : Increase in cell load of Isolate SRD 5 in presence of organic carbon supplement



In presence of glucose in iron oxidation medium, the generation time of isolate SRD 5 was 15.47 h. However, in control flask, 15.57 h generation time was noted (Table 4.6). This result indicates that in presence of glucose, isolate SRD 5 grows fast. This result hints at the heterotrophic nature of *Acidithiobacillus ferrooxidans* isolate. Furthermore, in 72 h, SRD 5 gave 4.7 generation in presence of glucose but slightly less generations i.e. 4.6 were observed in absence of glucose. Casein and sucrose gave 1% and 8% decrease in cell yield of the isolate. By addition of other supplements like casein, sucrose, fructose, citric acid and TSB, the generation time increased from 24 to 94 h and accordingly less growth rates ranging from 0.041 to 0.011 H^{-1} were observed. Hence, it is concluded that out of all organic substrates tested, only glucose supports better growth of SRD 5 in ferrous sulphate containing medium as compared to control flask having ferrous sulphate alone. This result contradicts the reports of obligate autotrophic nature of *Acidithiobacillus ferrooxidans*, which was found to be mixotrophic in our study.

Table 4.6 : Growth parameters of isolate SRD 5 in presence of organic carbon supplement in iron oxidation (inorganic) medium

Substrate	Generation time G (H)	No of generations in 72 h (n)	Growth rate R (H^{-1})
Glucose*	15.47	4.7	0.065
Control*	15.77	4.6	0.063
Mannitol*	15.93	4.5	0.063
Casein*	24.34	3.0	0.041
Sucrose*	25.33	2.8	0.039
Fructose*	34.16	2.1	0.029
Citric acid*	46.81	1.5	0.021
TSB*	93.21	0.8	0.011
Malic acid*	-86.64	-0.8	-0.012
Yeast extract*	-418.99	-0.2	-0.002

S.D. = 0.175 ± 0.003

* indicates all the treatments to be significantly different with respect to each other at $P<0.99$

5.1 INTRODUCTION

The solubilization of metals from low grade sulfidic ores by the activities of the chemolithotrophic bacterium *Acidithiobacillus ferrooxidans* is important to the mining industry, but an important side effect of bioleaching is co-dissolution of other heavy metal ions present in the ore. Waste products from the mining and metal refining industries often contain substantial amount of ferric iron, soluble heavy metals (e.g., arsenic, mercury, and silver), as well as unusually high concentrations of potentially toxic metals (e.g., copper and iron) (Schippers *et al.*, 2000). Tuovinen and Bhatti (1999) have reported that the pregnant solutions from underground stope bioleaching circuits contain as high as 2.5 to 10 g/l U_3O_8.

The heavy metal ions if accumulated to a certain level, it will deactivate or even kill the bacteria and eventually stop the activity of *A. ferrooxidans* cells (Dave *et al.*, 1979). The deactivation process may involve blocking or damaging of a significant fraction of the active sites on the microbial enzymes by heavy metal ions. On the contrary, Paknikar (1991) has reported that *Thiobacillus ferrooxidans* is also able to accumulate Ag, Cd, Co, Cu, Cr, Fe, Ni and U from solutions.

Leaching tanks used for the bio-oxidation of gold-bearing pyrite/arsenopyrite concentrates usually contain upto 0.5 M of dissolved iron. In the bio-heap reactors for bioleaching of low grade copper sulfide ore, the solution may also contain upto 0.4 M dissolved iron (Kawabe *et al.,* 2003). At such high concentrations of ferric iron, *A. ferrooxidans* is less important than a combination of *A. ferrooxidans* and *Leptospirillum ferrooxidans* combined (Dave, 2008). Many scientists have tried to understand the effect of high concentrations of ferric ions on ferrous ion oxidation rate and proved the results by mathematical model.

Earlier researchers observed that growth and Fe^{+2} oxidizing activity of *A. ferrooxidans* were decreased by the presence of 0.1 mg/l silver.

Tuovinen and Kelly (1974) studied the effect of UO_2^{+2} and found out that it partially inhibited the oxidizing activity of *A. ferrooxidans*. They also found that the addition of a complexing agent, such as EDTA or a non-toxic metal ion (e.g. Mg^{+2}) reduced the inhibitory action.

It has also been proved that mercuric and mercurous ions are more toxic to the bacteria than uranyl or other heavy metal ions like lead, arsenic, zinc, molybdenum, copper, cadmium and silver. Hence bioextraction can only be effective if the bacterium is resistant to the metal recovered as well as to others in the environment (Chisholm *et al.*, 1998; Kim *et al.*, 2005). Li and Ke (2001) reported that Cu^{+2} adapted strains of *Thiobacillus ferrooxidans* exhibited better leaching efficiency of nickel and copper from nickel bearing pyrrhotite.

Arsenic is a major impurity present in most sulfide ores. It is mainly present as arsenopyrite (FeAsS) in gold sulfide ore and concentrates and as energite (Cu_3AsS_4) and tennantite ($(Cu, Fe)_{12}As_4S_{13}$) in copper ores and concentrates. It can also be present as realgar (AsS) and orpiment (As_2S_3) in other metal sulfide as pyrite (Wierts *et al.*, 2006). Numerous arsenic-oxidising microorganisms, especially *Thiomonas* are able to oxidize As(III) to As(V) in order to detoxify their immediate environment (Moreira and Amils, 1997; Emerson and Moyer, 1997; Battaglia *et al.*, 2002; Bruneel, 2003; Chen *et al.*, 2004; Popa and Kinkle, 2004; Bruneel *et al.*, 2005; Duquesne, 2008; Bryan *et al.*, 2009).

A. ferrooxidans has a notably high tolerance for various heavy metals, ferric iron and acidity. In the present study, tolerance of an *A. ferrooxidans* isolate towards ferric iron and various metal and metalloids like copper, cadmium, zinc, nickel and arsenic was studied and isolates were adapted to resist higher concentrations of ferrous, ferric and arsenic.

5.1.1 MECHANISM OF RESISTANCE TO METALS

The variation in the toxicity of heavy metals might be due to differences in the mechanism of interaction between the bacteria and individual heavy metal ions. Exchangeability of Uranyl ion suggests the possibility of physical adsorption of UO^{+2} onto the cell and its easy exchange with a divalent cation like Mg^{+2}. On the other hand Hg^{+2} and Ag^{+} may more strongly interact with the ferrous ion oxidizing enzymes of *A. ferrooxidans* (Shao-yuan and Zhao-heng 2005; Yun-Guo *et al.*, 2007).

There have been many investigations to understand the mechanism of heavy metal resistance, but so far the studies are limited upto arsenic, mercury and to some extent copper (Felicio *et al.,* 2003). Tuovinen (1971) showed that *Thiobacillus ferrooxidans* when grown in the presence of Cu^{2+} ions showed varying extent of lag period depending upon the levels of copper present in 9K growth medium. The resistance of copper is induced after prolonged exposure to copper and it is not a permanent trait of the adapted strain (Das *et al.*, 1998). Alvarez and Jerez (2004) reported a model for copper detoxification in which 1 to 2 μM copper stimulated polyphosphate hydrolysis and the metal-phosphate complexes formed were transported out of the cell as part of a possibly functional copper tolerance mechanism in *A. ferrooxidans*.

Felicio *et al.* (2003) analyzed the total membrane protein profiles of *A. ferrooxidans* cells grown in presence and absence of 200 mM copper sulfate by 2D PAGE. Crude preparations of outer membrane and periplasmic proteins were analyzed by SDS-PAGE. The analysis showed over expression of a periplasmic protein about 17 KDa in copper adapted cells and it was assumed to be rusticyanin – a 16.5 KDa periplasmic copper protein present in *A. ferrooxidans* cells. Rusticyanin is involved in the electron transport chain of the iron oxidation pathway.

The resistance to arsenite by *A. caldus* was found due to energy dependent transportation of arsenate and arsenite out of the cell against a concentration gradient (Dopson *et al.*, 2001). Genome analysis confirmed the presence of a divergent gene cluster (AFE2857-60), which is known to be involved in arsenic resistance. The cluster includes genes encoding an arsenate reductase (ArsC), the arsenate repressor (ArsR), the divergently-oriented arsenate efflux pump (ArsB), and a hypothetical protein (ArsH). The *arsCRB* gene cluster was shown to confer resistance to arsenate, arsenite and antimony in *E. coli*, but the function of *arsH* is unknown (Butcher *et al.*, 2000).

Mercury resistance has been investigated in several strains of *A. ferrooxidans*. Genome analysis of the type strain (ATCC 23270) identified three genes potentially encoding the well-described Mer components, i.e., the repressor accessory protein (MerD), the mercury reductase (MerA), and the mercuric ion transporter (MerC). Four candidate genes potentially encoding members of the family of MerR-like transcriptional regulators were also found. The mechanism of growth inhibition by tungsten was attributed to the inhibition of the cytochrome-c oxidase activity in the plasma membrane (Felicio *et al.*, 2003).

The *A. ferrooxidans* genome and plasmid also contains several genes predicted to be part of heavy metal tolerance systems including genes for the copCD copper extrusion system, ten clusters of genes predicted to belong to the resistance-nodulation-cell division (RND) family of transporters, three genes encoding cation diffusion facilitator (CDF) proteins, three genes encoding copper translocating P-type ATPases, and two genes encoding other P-type ATPases of unknown specificity (Silver and Ji, 1993). These genome-based predictions offer new opportunities for experimental validation of heavy metal resistance in *A. ferrooxidans* and also provide new markers for detecting similar genes in other microorganisms.

A. ferrooxidans is resistant to heavy metals and metalloids at concentrations in the mg/L range, which is considered toxic for other microorganisms (Table 5.1) (Gomez *et al.*, 1999). Because of this property, bacterial leaching methods have been developed for the decontamination of soil and sediment polluted by heavy metal (Park *et al.*, 2007). De Siloniz *et al.* have reported growth of a new *Thiobacillus* isolate in presence of 500mM aluminium, 30mM arsenic and 50mM copper (De Siloniz *et al.*, 1993). *A. ferrooxidans* was reported to tolerate a very high concentration of Zn, Ni, Cu, Mn and Al (more than 10 g/L) but molybdate above 5 mg/L was toxic, while Venkatakrishna reported that it resisted molybdate upto 2.6 mM concentration (Venkatkrishna 2002).

Many strains of *A. ferrooxidans* viz. CC1, ATCC 23270, ATCC 33020, ATCC 19859 and BRGM1 were found to precipitate arsenite (As^{+3}) with ferric ion from the medium, specifically when the organism was grown on iron. *L. ferriphilum* Fairview was capable of growth and iron oxidation upto 60 mM As^{+3} and As^{+5}, while *L. ferrooxidans* ATCC49881 and *L. ferrooxidans* DSM2705 grew upto 40 mM As^{+3} and As^{+5} (Tuffin *et al.*, 2006).

The effect of heavy metals on ferrous iron oxidizing ability of *A. ferrooxidans* was studied by De *et al.* (1997) and showed that presence of some cations e.g. Hg^{+2}, Ag^{+}, Pb^{+2} and Cd^{+2} at a concentration level of 10 mg/l inhibits the microbial oxidation of Fe^{+2}. The presence of 10 mg/l of As^{+3}, Mn^{+2}, Sn^{+2}, Co^{+2}, Cu^{+2} and Zn^{+2} and anions such as Cl^{-} and NO_3^{-} did not have an observable effect on Fe^{+2} oxidizing activity of the bacteria (De *et al.*, 1997).

Table 5.1: Metal concentration tolerated by different *A. ferrooxidans* strains.

	Metal concentration (mM)				
Reference	**Copper**	**Nickel**	**Cadmium**	**Arsenite**	**Zinc**
Rawling 2005	220	274	-	-	668
Dopson 2003	800	1000	500	84	1071
Zakaria 2002	41	-	-	-	76
Novo *et al.*, 2000	200	600	600	-	600
Kupka & Kupsakova (1999)	<343	<233	-	-	-
Leduc *et al.* 1997	160	160	-	-	-
Huber *et al.* 1989	160	170			1201
Tuovinen *et al.*1974	500	500	-	-	-

5.2 MATERIALS AND METHODS

5.2.1 EFFECT OF INITIAL FERRIC IRON CONCENTRATION IN MEDIUM ON FERROUS SULPHATE BIOOXIDATION.

To study the comparison of microbial oxidation of ferrous sulfate with chemical oxidation done by biogenic ferric solution, 15 flask system was kept. For inoculum generation, SRD 5 culture was grown in ferrous iron containing medium upto >95% ferrous oxidation and then the cells were harvested as shown in appendix II. The final inoculum density was kept $5X10^8$ cells/ml. For biogenic ferric solution, after harvesting of cells from above medium, ferric settled in centrifuge tubes was pooled and mixed with 60 ml sterile distilled water at pH 1.9. The above solution was kept in boiling water bath for 30 minutes and cooled. Double strength 9K medium was made and sterilized separately. A set of 15 flasks was kept as shown in the table 5.2.

Table 5.2: Experimental design for influence of ferric ion study.

Falsk no.	$FeSO_4$ concentration	Inoculum	Additional biogenerated Fe^{+3}
1 – 5	5, 10, 15, 20, 25 %	10 %	-
6 – 10	5, 10, 15, 20, 25 %	10 %	10%
11 – 15	5, 10, 15, 20, 25 %	-	10%

5.2.2 ARSENITE AND ARSENATE RESISTANCE

The resistance of organisms towards various concentrations of arsenite and arsenate was studied. Growth medium used in the experiment was 9K medium. Potassium arsenate $KAsO_2$ and potassium arsenite KH_2AsO_4 were added in 90ml of above-mentioned medium in a range of 10 mM to 1000 mM concentrations and inoculated with actively growing 10% inoculum having cell load of $5X10^8$ cells/ml. One flask without arsenite/arsenate addition was used as positive control to compare the ferrous oxidation. The result was observed in terms of inhibition/delay of ferrous oxidation.

5.2.3 HEAVY METAL RESISTANCE

Heavy metal resistance was studied using 9K medium. The experiment was carried out in 250 ml flask having 50 ml system inclusive of 10% inoculum and highest reported concentrations of copper sulfate ($CuSO_4.5H_2O$), zinc sulfate ($ZnSO_4.H_2O$), nickel sulfate ($NiSO_4.6H_2O$), cadmium sulfate ($3CdSO_4.8H_2O$) and potassium arsenate ($KAsO_2$) in the literature. The salts when added in medium gave final concentrations as 865 mM and 802 mM of Cu^{+2} ion, 1.83 M and 1.07 M of Zn^{+2} ion, 1.23 M and 1.0 M of Ni^{+2} ion, 498 mM and 400 mM of Cd^{+2} ion and 84 and 40 mM of As^{+5} ions in individual flasks. One 9K medium containing flask without any metal addition

was inoculated and incubated at same conditions to serve as positive control.

All the flasks were kept on environmental shaker and incubated at 30±2°C at 150 rpm. At every 24 hrs. interval, samples were drawn aseptically for cell count and remaining ferrous iron estimation in medium, as per appendix II. The result was observed in terms of inhibition/delay of ferrous oxidation.

5.3 RESULTS AND DISCUSSION

5.3.1 EFFECT OF INITIAL FERRIC IRON CONCENTRATION IN MEDIUM ON FERROUS SULPHATE BIOOXIDATION.

To find out effect of additional ferric, oxidation of 5% to 25% ferrous sulphate by isolate SRD 5 was studied in presence and absence of 10% additional biogenerated ferric. This experiment is particularly important for bacterial monoculture/consortium being developed for bioleaching of metals, because during leaching of metal sulphide concentrates, the dissolved iron concentration in leachate rises upto 400 mM to 500 mM (Kawabe *et al.*, 2003). The isolate developed for leaching should be resistant to this much high concentrations of iron.

As can be seen from data presented in table 5.3, at 5% $FeSO_4$ concentration, control flask having inoculum but no additional ferric gave an IOR of 0.398 g/L/h as against 0.351 g/L/h IOR in presence of 10 % ferric and inoculum. This indicates that additional ferric iron present in the system has marginal negative effect on iron oxidation. More than 99% oxidation in these two flasks had completed within 2 days. In the flask having only ferric sulphate and no inoculum, IOR as low as 0.043 g/l/h was observed and ferrous iron was not completely oxidized even on 10^{th} day of incubation.

Table 5.3 : Inhibition of iron oxidation at different ferrous iron concentration

	Ferrous sulphate concentration				
(%)	**5%**	**10%**	**15%**	**20%**	**25%**
(mM)	100	200	300	400	500
	Iron oxidation rate (g/l/h)				
No ferric, 10 % inoculum	0.398	0.271	0.240	0.163	0.041
10 % ferric, 10 % inoculum	0.351	0.271	0.245	0.180	0.041
10 % ferric, No inoculum	0.043	0.081	0.060	0.074	0.032
	Days taken for >99% iron oxidation				
No ferric, 10 % inoculum	2	3	6	10	-
10 % ferric, 10 % inoculum	2	3	6	10	-
10 % ferric, No inoculum	10	10	20	22	-
	Percent decrease in IOR due to presence of higher concentration of ferrous sulphate				
No ferric, 10 % inoculum	0	32	40	59	90
10 % ferric, 10 % inoculum	12	32	38.5	54.8	90

During study of 5% ferrous sulphate oxidation, control flask inoculated with iron oxidizing isolate SRD5, but not having in additional ferric showed 94.5% iron oxidation in 1 day itself, while the flask having additional 10% ferric showed 83.4% oxidation (Graph 5.1).

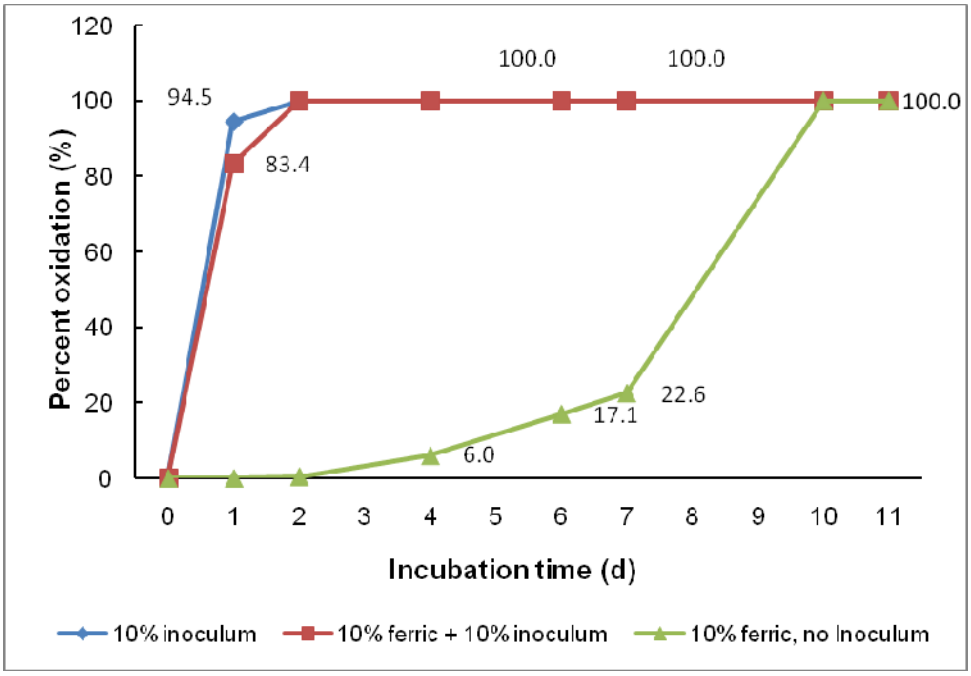
When the effect was observed on 10% ferrous sulfate oxidation (Graph 5.2), both the flask having presence and absence of 10% ferric iron showed IOR of 0.271 g/L/h. After 24 h, in control flask having no additional ferric, 36.9% iron oxidation was completed as compared to 34.4% iron oxidation in flask having 10% additional ferric. Hence the detrimental effect of ferric is visible on test culture. However after 2 days, same amount of iron was oxidized and both the flasks showed more than 99% oxidation in 3 days. In the flask having only ferric sulphate and no inoculum, IOR increased to 0.081 g/l/h as compared to 0.043 g/l/h in 5% $FeSO_4$ containing flask. Here also >99% ferrous iron oxidation was achieved in 10 days.

However isolate showed 32% decrease in IOR, i.e 0.271 g/l/h, while oxidizing 10% ferrous sulphate as compared to 0.398 g/l/h IOR in presence of 5% ferrous sulphate as energy source (Table 5.3).

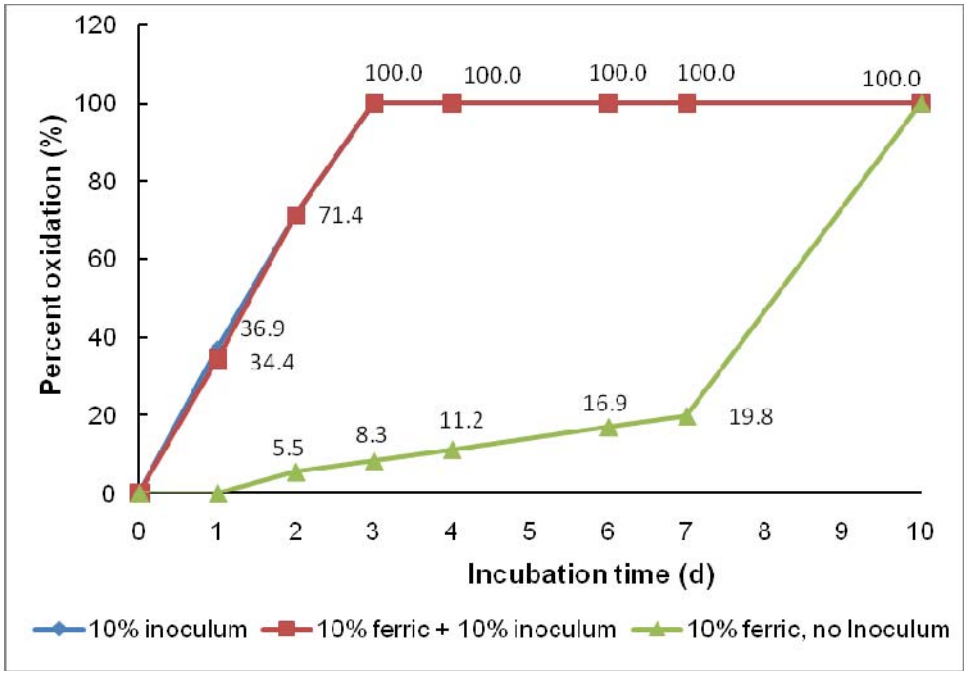
During the experiment with 15% ferrous sulfate, flask having 10% additional ferric showed 0.245 g/l/h IOR, while flask having only inoculum showed less IOR i.e. 0.240 g/l/h (Graph 5.3). The same pattern was observed during 20% ferrous sulfate oxidation, where higher oxidation rate, i.e. 0.180 g/l/h was observed in flask having 10% additional ferric (Graph 5.4). These flasks took 6 days and 10 days for oxidation of 15% and 20% ferrous sulfate respectively (Table 5.3).

In inoculated flasks having 15% ferrous sulphate, the rate of iron oxidation by isolate had decreased by 40% as compared to 5% ferrous sulphate oxidation and the IOR still decreased by 69% during oxidation of 20% ferrous sulphate.

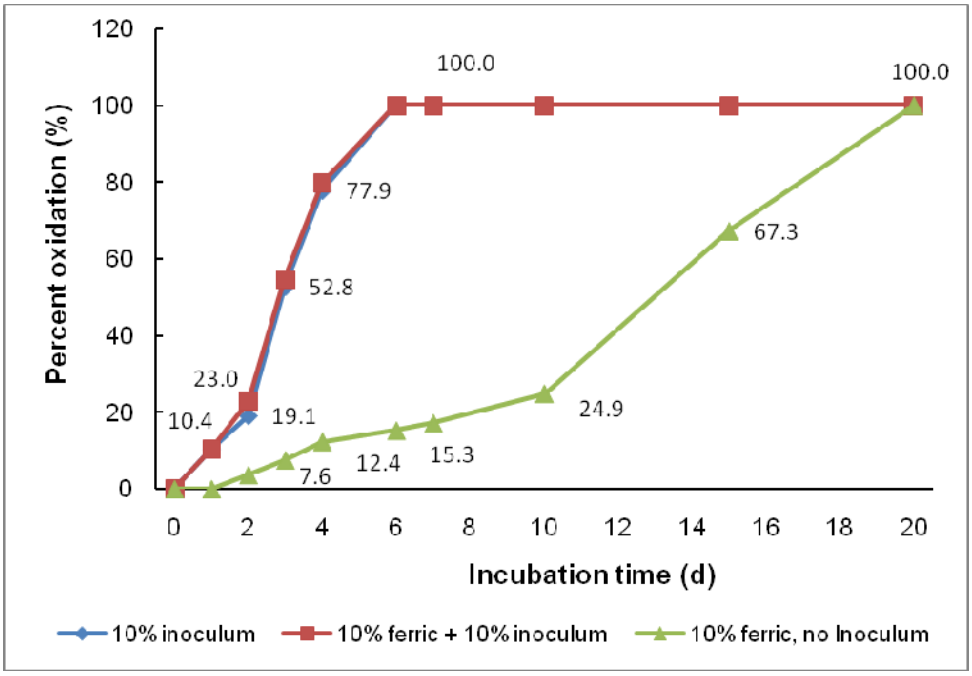
Graph 5.1 : Percent iron oxidation in presence of 5% ferrous sulfate



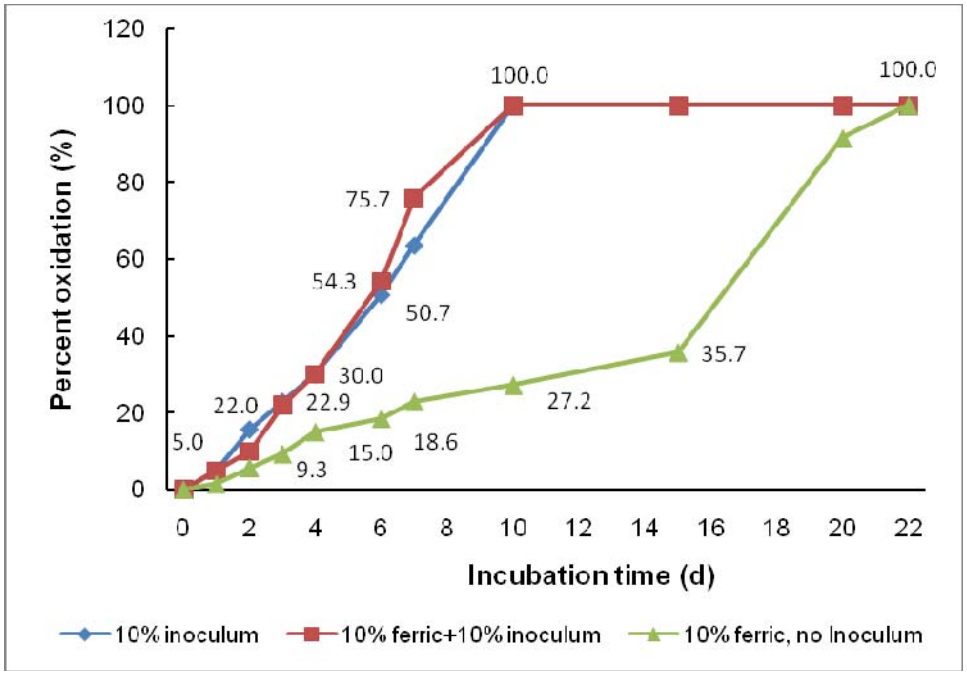
Graph 5.2 : Percent iron oxidation in presence of 10% ferrous sulfate



Graph 5.3 : Percent iron oxidation in presence of 15% ferrous sulfate



Graph 5.4 : Percent iron oxidation in presence of 20% ferrous sulfate



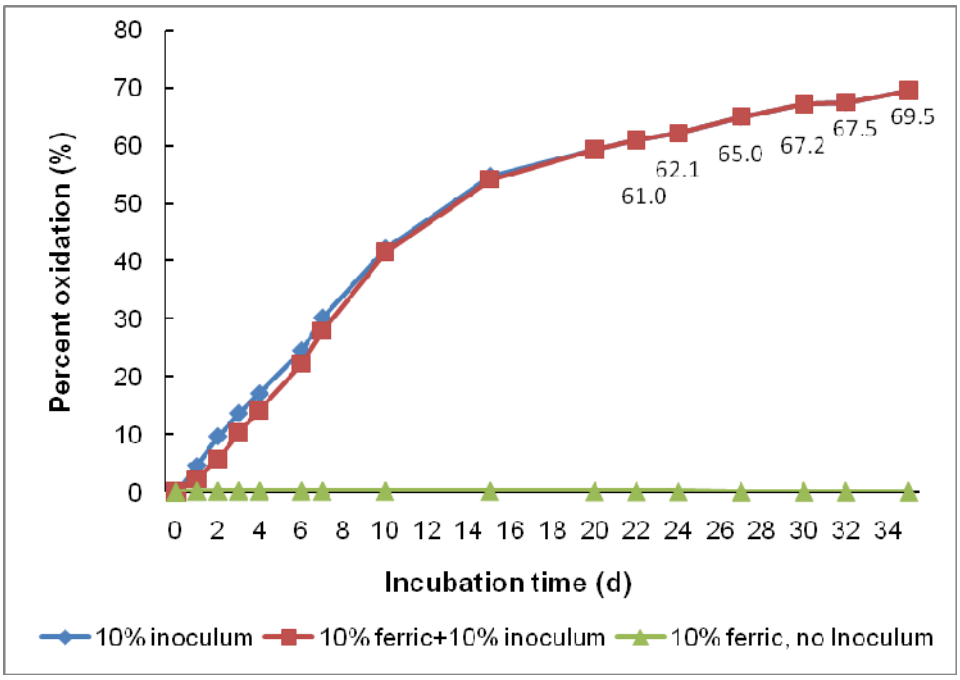
Even though the isolate was able to completely oxidize 15% and 20% ferrous sulfate in 6 and 10 days respectively, it could not oxidize 25% ferrous sulfate even after 35 days (Graph 5.5). With 25% $FeSO_4.7H_2O$ concentration, *A. ferrooxidans* was able to oxidize only 69.5% ferrous in 35 days in absence and presence of 10% ferric iron. Hence it is understood that the organism is able to resist 20% ferrous sulfate or 400 mM (Fe^{+2}), but in presence of 500 mM (Fe^{+2}), iron oxidation becomes very slow and almost 90% decrease in iron oxidation is observed. At the end of the experiment with 25% $FeSO_4.7H_2O$, only 7.5 x 10^4 cells/ml had survived as against the initial cell load of 5 x 10^8 cells/ml.

Based on the iron oxidation rates given by each of the three combinations of inoculum and biogenerated ferric, graph 5.6 was plotted. As shown in graph 5.6, inoculated flask having no ferric iron, show rapid decrease in IOR of 5% to 25% ferrous sulphate. Presence of 10% ferric in the inoculated medium results in slow decrease of IOR from 5% to 25% ferrous sulphate. Moreover, inoculated 9k medium having no ferric iron gives better IOR in 5% ferrous sulfate containing medium as compared to ferric iron and inoculum combined.

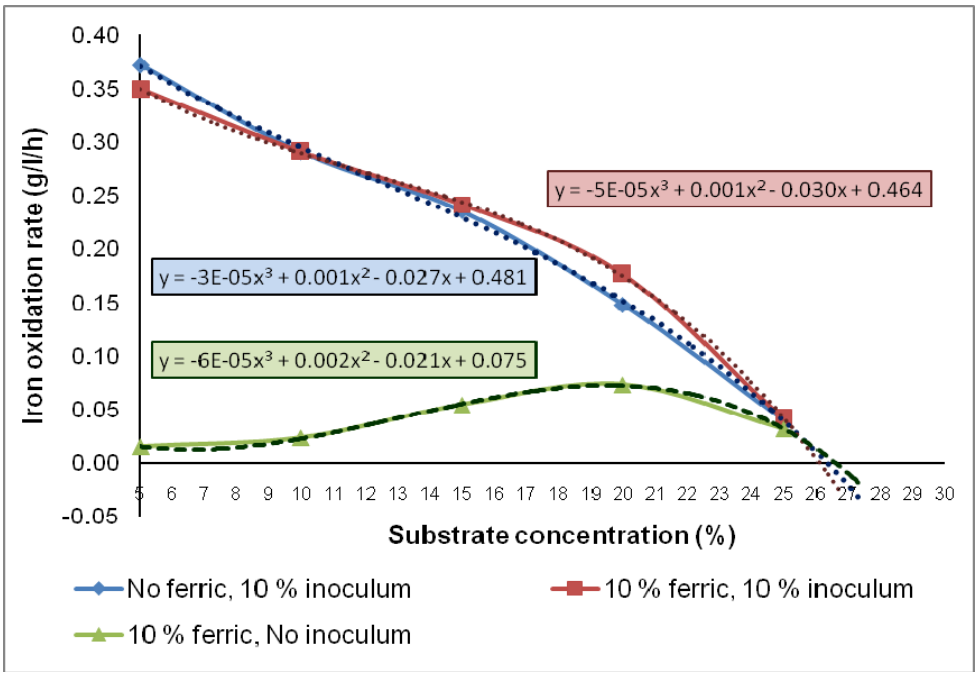
If polynomial trendlines for each of the three combinations are drawn, as shown in graph 5.6, it is most identical to the graph. This trendline gives us an equation exactly depicting the pattern of observed graph. In equation of all these lines, if value of Y is kept '0', we get the value of 'X', i.e. the concentration of ferrous sulphate, where IOR will be '0'.

By this calculation, inhibitory concentration of ferrous was calculated for each of the three combinations: A: no ferric, 50 % inoculum, B: 10 % ferric, 50 % inoculum and C: 10 % ferric and no inoculum. It was found to be A – 26.3%, B- 26% and C – 26.6% ferrous sulphate. It means that in absence and presence of 10% ferric iron, ferrous iron oxidation by inoculated culture SRD5 will be completely inhibited at 26.3% and 26% ferrous sulfate, which is equivalent to 526 and 520

Graph 5.5 : Percent iron oxidation in presence of 25% ferrous sulfate



Graph 5.6 : Effect of inoculum and ferric iron on IOR



	Inhibition of ferrous oxidation at	
	Fe (%)	Fe (mM)
No ferric, 10 % inoculum	26.3	526
10 % ferric, 10 % inoculum	26	520

mM ferrous iron. This observed oxidation by our isolate SRD5 is better than the results described for ATCC 19859 culture, where Kawabe *et al*. reported complete inhibition of iron oxidation activity by *A. ferrooxidans* ATCC 19859 at 300 mM (15%) ferric ion concentration, while another isolate T23-3 could oxidize ferrous ion in presence of 480 mM ferric. The activity of strain ATCC 23270 decreased about 95% at 500 mM ferric (~25% FeSO4), while that of strain T23-3 decreased about 80% at 480 mM ferric (Kawabe *et al.*, 2003).

5.3.2 ARSENITE AND ARSENATE RESISTANCE

When arsenate ion (As^{5+}), in the form of potassium arsenate ($KAsO_2$) was added in medium at concentrations ranging from 20 mM to 100mM, its varied effect on iron oxidation by SRD 5 was observed. The ore isolate was able to resist 100 mM arsenate ion (As^{+5}) added as potassium arsenate in its first exposure itself (Graph 5.7). Upto 40 mM potassium arsenate in ferrous oxidation medium did not have any negative effect on iron oxidation and the iron oxidation in presence of 20 mM and 40 mM arsenate was similar to control flask i.e. 0.785 g/l/h (Table 5.4). However in presence of 60 mM arsenate in the medium, isolate SRD 5 showed 13% decrease in IOR from 0.785 g/l/h to 0.684 g/l/h. The effect of arsenate was more clearly visible at 80mM and 100 mM concentrations. At these 2 concentrations iron oxidation was completed in 4 days and the IOR decreased by 28% and 32% respectively, as compared to control flask.

Once the isolate was able to grow and oxidize iron in presence of 100mM arsenate, the cells from 100mM arsenate containing flasks were harvested on completion of experiment and exposed to higher concentrations of arsenate upto 1000mM. Ferrous oxidation by SRD 5 in presence of 100 – 1000 mM potassium arsenate (As^{+5}) is shown in graph 5.8. On second exposure to 100mM arsenate, the iron oxidation rate of isolate SRD 5 in presence of 100mM arsenate was similar to control flask i.e. 0.785 g/l/h.

Graph 5.7: Ferrous oxidation by SRD 5 in presence of 0-100mM potassium arsenate (As^{+5})

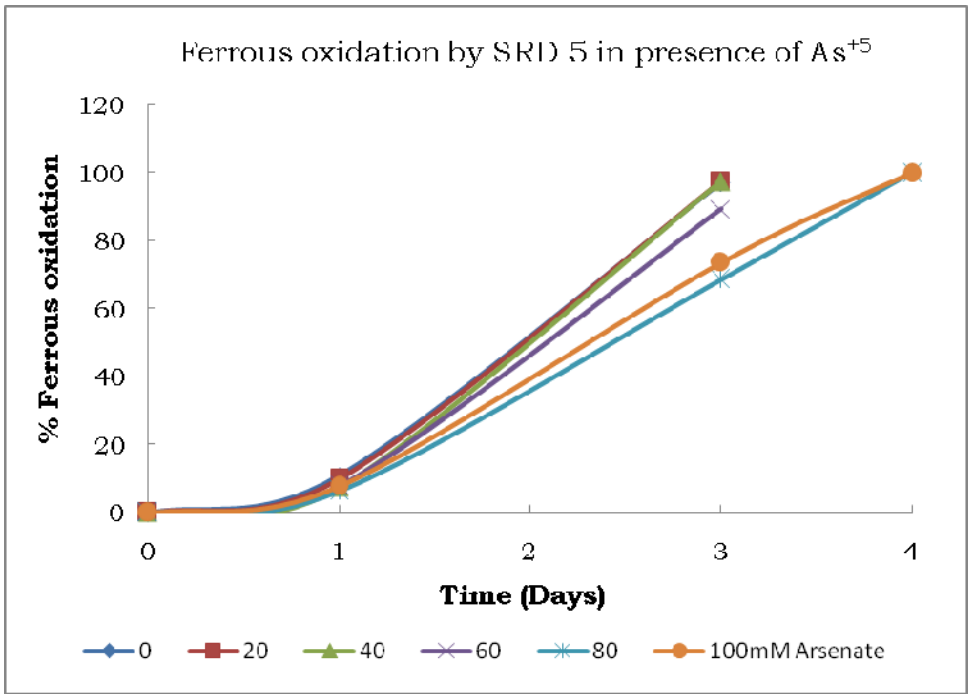


TABLE 5.4 : Percent decrease in IOR with 20-100 mM arsenite - SRD 5.

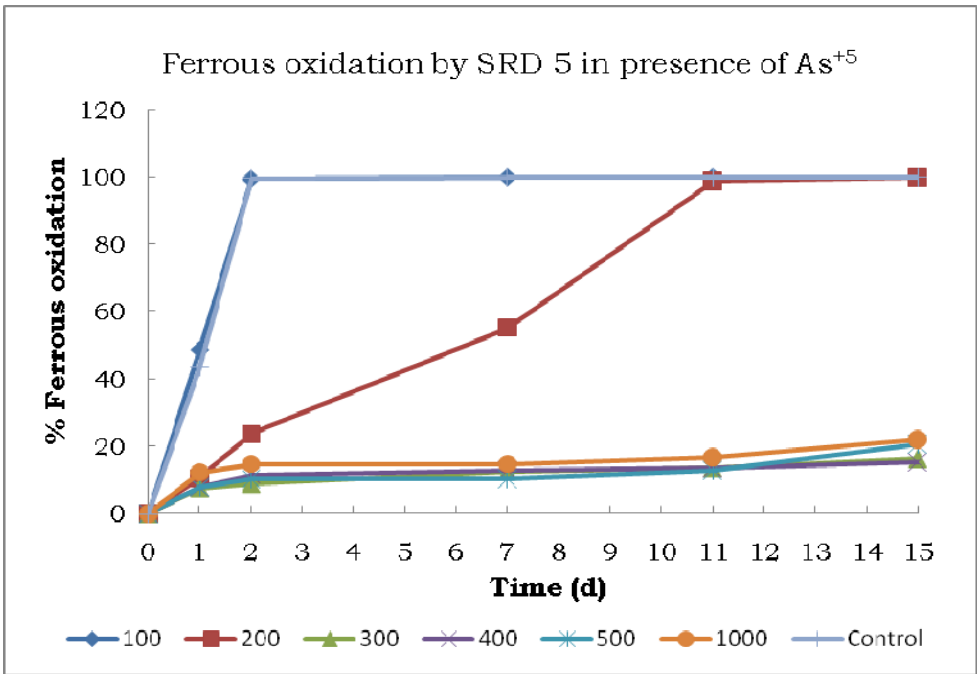
As^{+5} or As^{+3} conc. (mM)	Iron oxidation rate (g/l/h)		Days taken for 100% iron oxidation		% IOR		% decrease in IOR	
	As^{+5}	As^{+3}	As^{+5}	As^{+3}	As^{+5}	As^{+3}	As^{+5}	As^{+3}
Control	0.785	0.785	3	3	100	100	0	0
20	0.785	0.571	3	4	100	73	0	27
40	0.785	0.556	3	4	100	71	0	29
60	0.684	0.200	3	11	87	25	13	75
80	0.564	0.114	4	22	72	15	28	85
100	0.532	0.089	4	27	68	11	32	89

It indicated that isolate had adapted to 100mM arsenate. However in presence of 200mM arsenate, the observed IOR was 0.307 g/l/h, i.e. 39% IOR was noted as compared to 100mM arsenate. Moreover oxidation of ferrous took 5 days to complete. However ferrous oxidation in flasks containing 300mM to 1000mM arsenate were not completed even after 22 days and iron oxidation rate had decreased to as low as 0.03 g/l/h in those flasks. The above results indicated that iron oxidation by SRD5 is inhibited at 300mM potassium arsenate concentration.

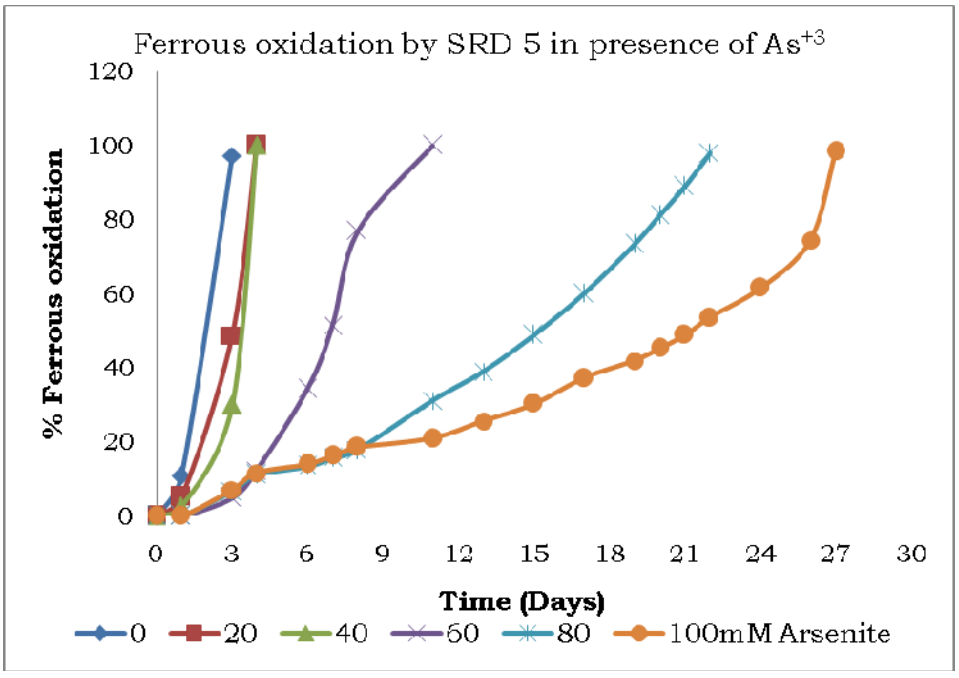
De Siloniz *et al.* (1993) have reported growth of a new *Thiobacillus* isolate in presence of 30mM arsenate. Cassity and Pesic (1999) reported that arsenate ion had positive effect on the rate of Fe^{+2} oxidation in short term kinetic studies (up to 30 minutes). This is explained by further reaction between Fe^{+3} and As^{+5}, which forms ferric arsenate precipitate. Removal of these precipitate stimulated further oxidation of Fe^{+2} causing increase in ferrous oxidation rate. However sodium arsenite at 2.67 mM concentration did not affect the steady state growth kinetics or cell yield in *A. ferrooxidans* (Braddock *et al.*, 1984)

When iron oxidation by SRD 5 was studied in presence of 0 to 100mM potassium arsenite (KH_2AsO_4), decrease in IOR was observed at all concentration of potassium arsenite tested (Graph 5.9). Control flask showed 0.785 g/l/h IOR. While flasks having 20mM and 40mM arsenite showed 0.571 g/l/h and 0.556 g/l/h IOR. This accounted to 27% and 29% decrease respectively (Table 5.4). However in flasks having 60 mM, 80 mM and 100 mM As^{+3}, lag phases of 4, 8 and 10 days were seen, after which the IOR increased. As a result, SRD5 took 11, 22 and 27 days to completely oxidize 10% ferrous in presence of 60mM, 80mM and 100mM arsenite respectively. When the total incubation was considered, the IOR achieved in presence of 60mM, 80mM and 100mM As^{+3} were as low as 0.2, 0.114 and 0.089 g/L/h respectively.

Graph 5.8 : Ferrous oxidation by SRD 5 in presence of 100 - 1000mM potassium arsenate (As^{+5})



Graph 5.9 : Ferrous oxidation by SRD 5 in presence of 0-100 mM potassium arsenite (As^{+3})

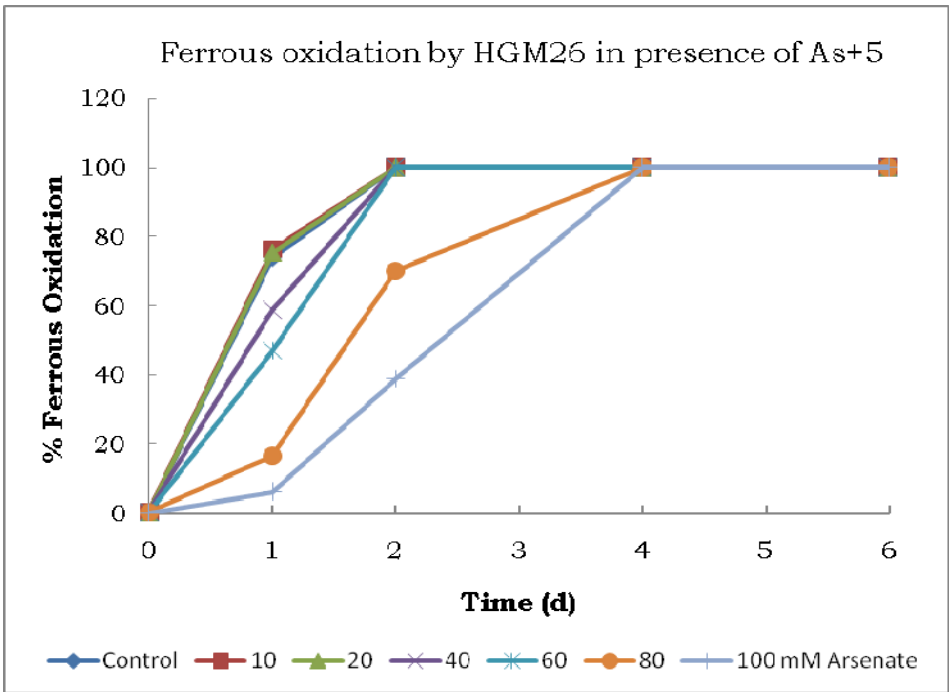


The same result was observed by Cassity and Pesic (1999), where addition of 1.5 g/l of As^{+3} had a negative effect on bioleaching of arsenopyrite ore by increasing the lag period. Only after most of the initial arsenite had been removed from solution did bioleaching enter the exponential phase.

Iron oxidation by HGM 26 was also studied in presence of 10mM to 1000 mM potassium arsenate ($KAsO_2$). In arsenate concentrations from 10mM to 60mM, the iron oxidation was completed in 2 days (Graph 5.10). At 10mM and 20mM arsenate concentration, very little decrease in iron oxidation rate was observed which was in range of 2-3% (Table 5.5). In these 2 flasks the IOR was 0.249 g/l/h and 0.246 g/l/h respectively, as compared to 0.254 g/l/h IOR obtained in control flask. At 40mM and 60mM arsenate concentration, 34% and 33% decrease in IOR was observed, which was 0.169 and 0.171 g/l/h respectively. However ferrous oxidation took 4 days in 80mM and 100mM arsenate flasks and 65%-66% decrease in IOR was observed.

In presence of arsenate concentration from 200mM to 1000mM, ferrous oxidation became very slow and it was not over even in 17 days (Graph 5.11). The flasks showed only 72% to 25% ferrous oxidation in 200mM, 400mM, 600mM, 800mM and 1000mM arsenate containing flasks. The iron oxidation rate fell below 0.015 g/l/h, which amounted to 94-98% decrease in IOR.

Graph 5.10 : Ferrous oxidation by HGM 26 in presence of 0-100mM potassium arsenate (As^{+5})



Graph 5.11 : Ferrous oxidation by HGM 26 in presence of 100-1000mM potassium arsenate (As^{+5})

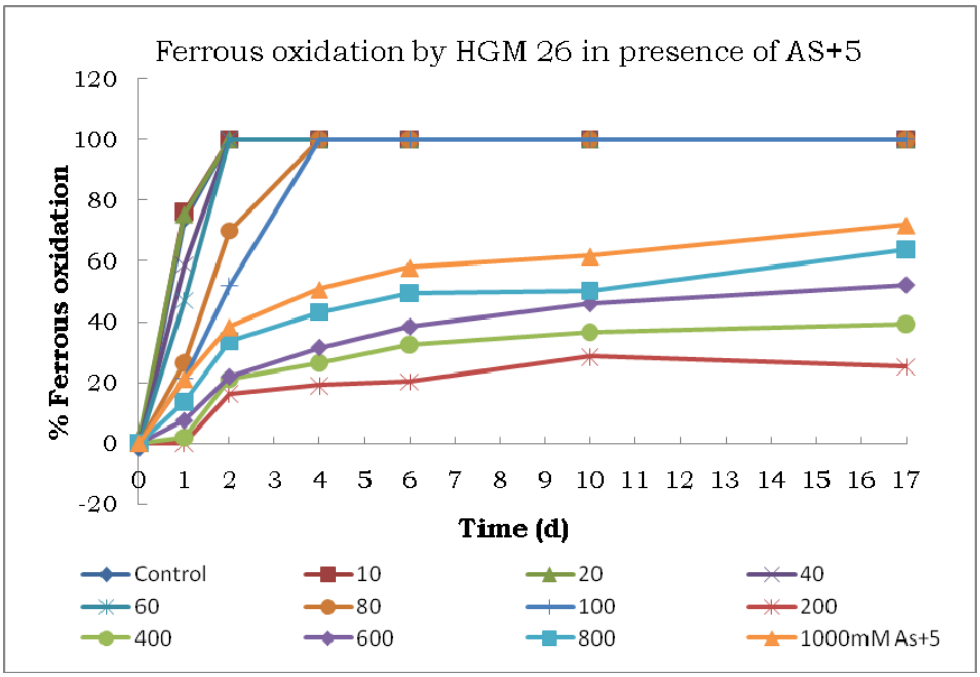


Table 5.5 : Effect of 10mM to 1000mM potassium arsenate (As^{+5}) on iron oxidation by HGM 26

As^{+5} conc. (mM)	Iron oxidation rate (g/l/h)	Days taken for 100% iron oxidation	% IOR	% decrease in IOR
Control	0.254	2	100	0
10	0.249	2	98	2
20	0.246	2	97	3
40	0.169	2	66	34
60	0.171	2	67	33
80	0.089	4	35	65
100	0.086	4	34	66
200	0.014	-	6	94
400	0.013	-	5	95
600	0.010	-	4	96
800	0.008	-	3	97
1000	0.005	-	2	98

5.3.3 HEAVY METAL RESISTANCE

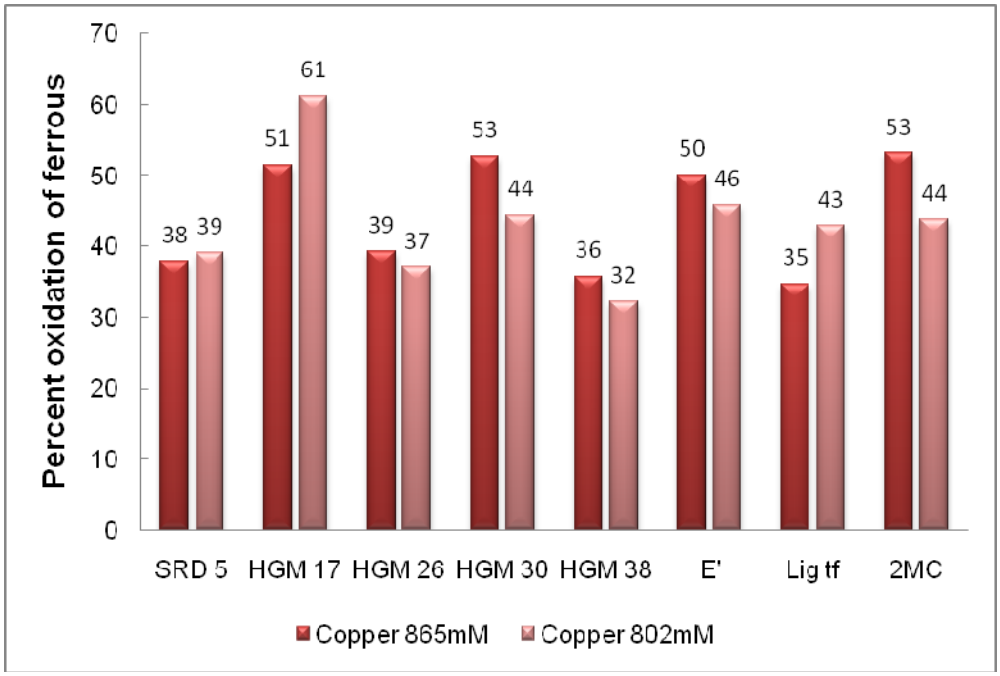
A. ferrooxidans is more resistant to heavy metals and metalloids as compared to other microorganisms. Venkatkrishna (2002) reported that *A. ferrooxidans* tolerated more than 10 g/L concentration of Zn, Ni, Cu, Mn and Al. Tuffin *et al.* (2006) reported that many strains of *Thiobacillus ferrooxidans* viz. CC1, ATCC 23270, ATCC 33020, ATCC 19859 and BRGM1 were found to precipitate arsenite (As^{+3}) with ferric ion from the medium, specifically when the organism was grown on iron. Kim *et al.* (2005) reported that the efficiency of cadmium (Cd) solubilization in the bioleaching process was 80%, which was higher than that of nickel (Ni) solubilization i.e. 60%.

Felicio *et al.* (2003) investigated the response of unadapted *A. ferrooxidans* –LR cells to nickel, zinc and cadmium sulfates. The time required by the bacterial cells to complete iron oxidation in the presence of 600 mM nickel sulfate, 600 mM zinc sulfate or 600 mM cadmium sulfate was about 120, 160 and 190 hrs.

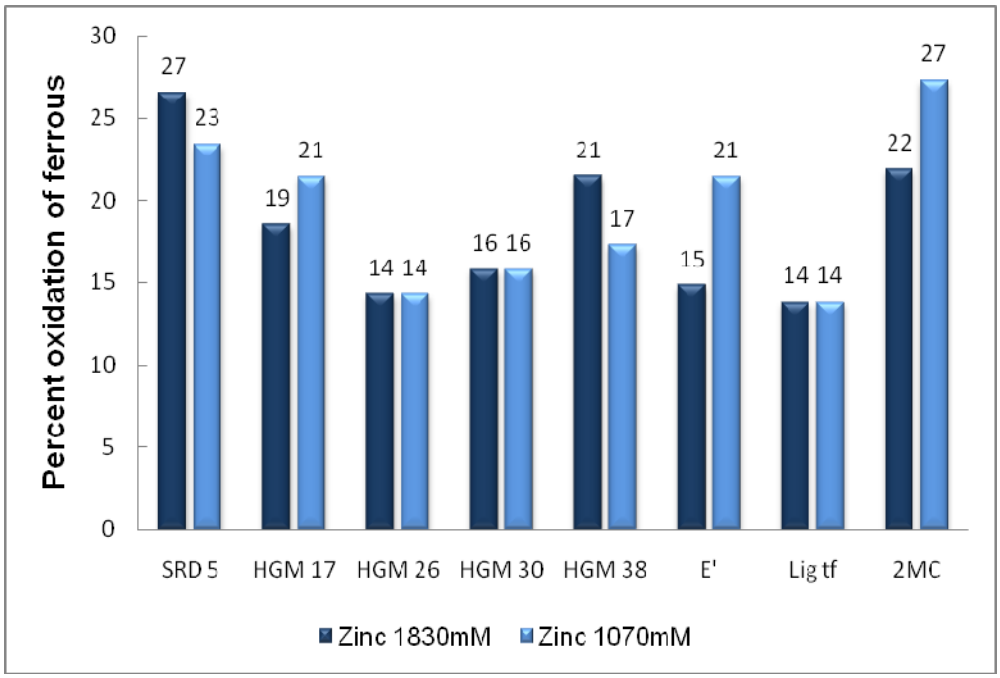
Resistance of all the eight isolates against copper, nickel, cadmium, zinc and arsenic ions at two different concentrations was studied. The isolates showed 32 to 61% iron oxidation in presence of 802 mM copper added as copper sulfate in 9K medium containing ferrous sulfate, while 35 to 53% iron oxidation was observed in medium containing 865mM copper (Graph 5.12). Isolate HGM 38 was the least resistant among all the isolates, while isolate HGM 17 showed highest resistance and remained active even at 865 mM copper and gave 51% iron oxidation. The resistance shown by our isolates is much higher than reported by Kupka and Kupsakova (1999), who reported 84% reduction in IOR of *T. ferrooxidans* in presence of 63mM $CuSO_4$. In their report, iron oxidation was completely inhibited at 343 mM copper and 233 mM nickel sulfate. Felicio *et al.* (2003) reported that out of copper, nickel, zinc and cadmium sulfates, copper sulphate showed the highest inhibitory effect on unadapted *A. ferrooxidans*–LR cells.

When iron oxidation in presence of 1070 mM and 1830 mM zinc was studied, our isolates showed only 14 to 27% iron oxidation in presence of both the concentrations tested (Graph 5.13). Isolates Lig-tf and HGM 26 were most affected and both of them gave only 14% iron oxidation. Isolate 2MC gave the maximum 27% iron oxidation at 1070 mM zinc and SRD 5 showed 27% iron oxidation of 1830 mM zinc.

Graph 5.12 : Percent iron oxidation by 8 isolates in presence of 865 mM and 802 mM copper



Graph 5.13 : Percent iron oxidation by 8 isolates in presence of 1830 mM and 1070 mM zinc

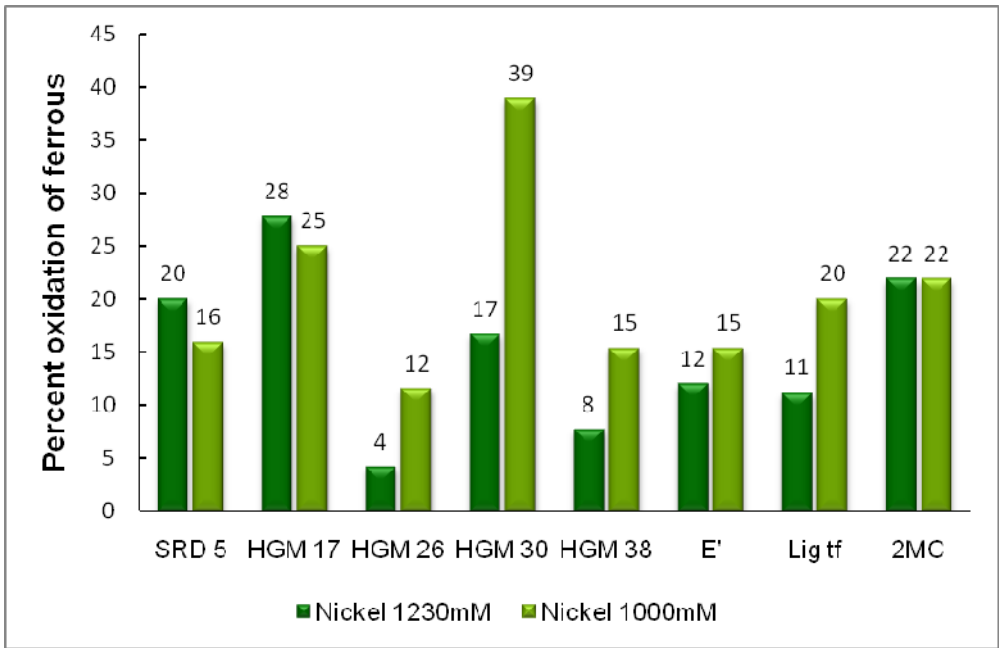


Resistance of all the eight isolates to nickel was also lower as compared to copper. Here isolates showed 4% to 28% iron oxidation in 1230 mM nickel sulphate containing flasks (Graph 5.14), while 12% to 39% iron oxidation was observed in 1000mM nickel containing flasks. Highest iron oxidation in presence of 1000 mM nickel was achieved by isolate HGM 30, which was followed by isolate HGM 17, which gave 25% and 28% iron oxidation in presence of 1000 mM and 1230 mM nickel respectively. Isolates HGM 26 and HGM 38 were most affected by higher concentration of nickel (1230 mM nickel) as only 4% and 8% iron oxidation was completed in 21 days.

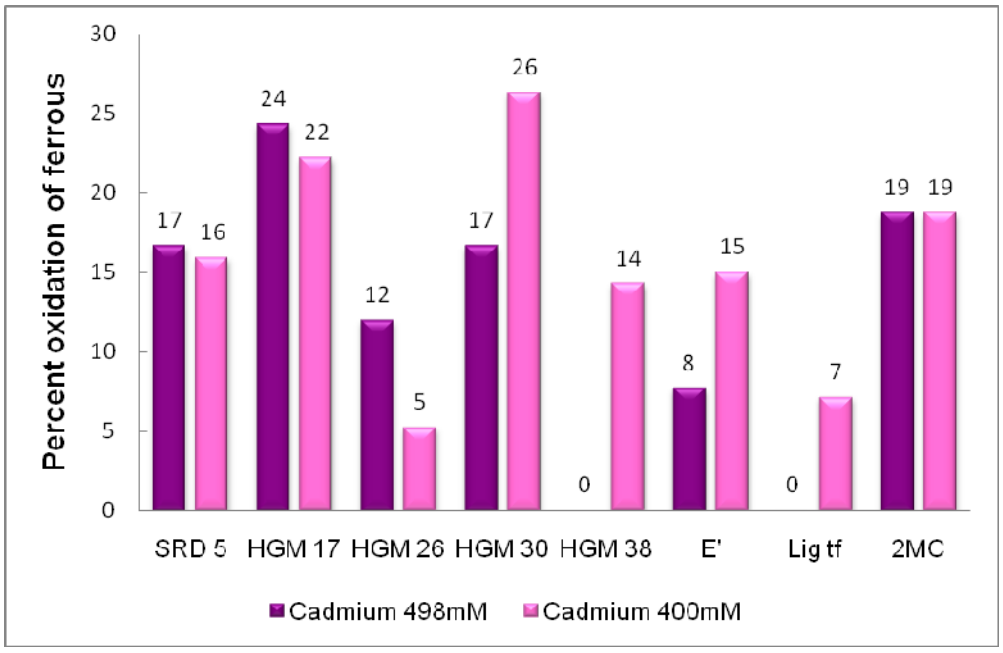
In presence of 498 mM cadmium, iron oxidation by all the isolates was in the range of 0-24% only (Graph 5.15). Two isolates HGM 38 and Lig-Tf, could not tolerate 498mM cadmium at all, because no iron oxidation was achieved by these 2 isolates even after 21 and 25 days respectively. Out of the 8 isolates tested for resistance against 400mM cadmium, highest iron oxidation (26%) was shown by HGM 30. All the isolates tested, showed 5% to 26% iron oxidation in presence of 400mM cadmium. Isolate HGM 17 also showed good resistance to cadmium and completed 22% and 24% oxidation of iron in presence of 400mM and 498 mM cadmium respectively.

As shown in graph 5.16, isolates HGM 17, HGM 26 and HGM 38 gave very good resistance against arsenic. This could be due to the fact that these cultures have been isolated from Hutti gold mine. Isolate HGM 26 completed >99% iron oxidation in presence of 40 mM and 84 mM arsenite. Moreover >99% iron oxidation was also achieved by isolate HGM 38 and HGM 17 in presence of 40 mM and 84 mM arsenite respectively. This may be due to the fact that gold mine has abundance of arsenopyrite, which releases arsenic in the environment as a result of bioleaching. Hence, these isolates have already been exposed to arsenic containing environment and therefore adapted to high content of arsenic.

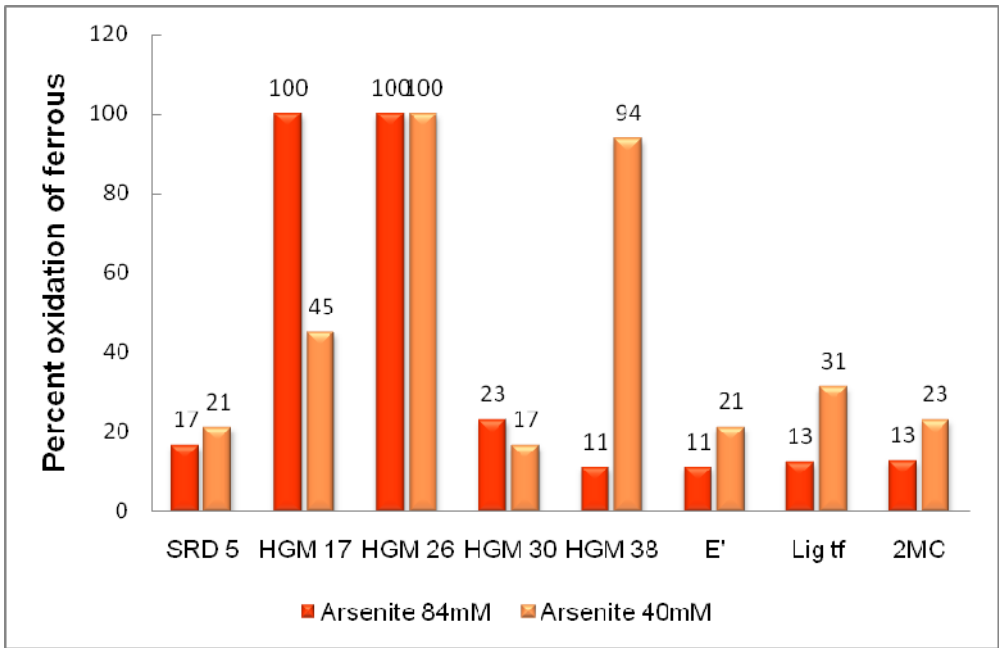
Graph 5.14 : Percent iron oxidation by 8 isolates in presence of 1230 mM and 1000 mM nickel



Graph 5.15 : Percent iron oxidation by 8 isolates in presence of 498 mM and 400 mM cadmium



Graph 5.16 : Percent iron oxidation by 8 isolates in presence of 84 mM and 40 mM arsenite



6.1 INTRODUCTION

6.1.1 MICROBIAL PYRITE OXIDATION

Iron sulfide ores, which are associated with bituminous coal mines or with metal sulfide deposits, are subjected to bacterial attack when in contact with aerated water. The reaction can be summarized as follows (Ehrlich, 1962; Mandl *et al.*, 1999; Kock and Schippers, 2006):

$2FeS_2 + 2H_2O + 7O_2 \rightarrow 2FeSO_4 + 2H_2SO_4$.....(1)

Once the pyrite oxidation has started- Eh, pH, dissolved Fe^{+2}, total iron, cell count, electrochemical noise (EN) i.e. spontaneous fluctuations of potential or current noise of electrochemical systems are checked to quantify the extent of pyrite oxidation (Bevilaqua, 2006).

Mustin *et al.* (1992) observed that pyrite oxidation takes place stepwise. These steps can be related to chemical (leachate content), bacterial and physical corrosion pattern sequentially. *A. ferrooxidans* and perhaps other acidophilic iron oxidizing bacteria attack the crystal lattice of the FeS_2.

FeS_2 is also oxidized chemically by Fe^{+3} (Lindstrom *et al.*, 1992):

$FeS_2 + 14\ Fe^{+3} + 8H_2O \rightarrow 15Fe^{+2} + 2SO_4 + 16H^+$.....(2)

The ferrous iron produced in reaction 2 is biologically oxidized to ferric (Fe^{+3}) as shown in reaction 3. This biologically generated ferric is again used for chemical oxidation of FeS_2 as shown in reaction 2. Fe^{+2} produced in reaction 1 as $FeSO_4$ may be further oxidized to ferric hydroxide according to reaction 3. It can be seen that acid is generated in this reaction.

$2Fe^{+2} + ½\ O_2 + 5H_2O \rightarrow 2Fe(OH)_3 + 4H^+$.....(3)

At high SO_4 concentration in the solution some of the ferric iron may be transformed into basic ferric sulphate or Jarosite as summarized in the equation 4:

$A^+ + 3Fe(OH)_3 + 2SO_4^{-2} + 3H^+ \rightarrow A.Fe(SO_4)_2(OH)_6 + 3H_2O$……(4)
(A^+ may represent either K^+, NH^{+4} or H_3O^+)

Analysis of bacterial population on pyrite surfaces over the time suggest that pyrite oxidation is dependent mainly on the fraction of bacteria bound to pyrite surface (Lindstrom *et al*, 1992; Hao *et al.*, 2006). Some moderately thermophilic bacteria inhabit sulfidic ore containing ecosystems and they are receiving more attention due to promising results in mineral sulfide bioleaching and biooxidation of arsenopyrite (Foucher *et al.*, 2003).

6.1.2 ARSENOPYRITE OXIDATION

Arsenopyrite is a recalcitrant mineral, in which gold is entrapped as submicroscopic particle in a matrix of iron and sulphur (Rawlings, 1998; Nagaoka *et al.*, 1999). Pyrite and arsenopyrite are concentrated from ore by floatation process (Natarajan, 1988). For extraction of gold, pretreatment of the mineral is necessary to breakdown the sulphide matrix. Roasting and pressure oxidation are well known pretreatment approaches for liberating gold from refractory ores and concentrates. Although they have become commercially established techniques, their application is restricted by high capital investment and operating cost as well as environmental regulations (Liu *et al.*, 1991; Rawlings, 1998). A promising approach to solve this problem is the microbial leaching of the arsenopyrite containing gold mineral. Here, microbes are used to breakdown the matrix because they oxidize some components of the arsenopyrite and open the structure.

Cyanidation is most common commercial process for recovery of gold after pretreatment. The matrix of arsenopyrite and pyrite protects gold from making soluble complex with cyanide (Gardner and Rawlings, 2000). Once the matrix is broken by microbial activity, it allows gold solubilizing chemicals such as cyanide to penetrate the mineral and accelerate gold solubilization (Rawlings *et al.*, 2003).

Additional costs are incurred for the safe disposal of the highly toxic cyanide and arsenic trioxide products.

In a mixture of pyrite and arsenopyrite, bacterial leaching proceeds in 2 consecutive phases. During first phase, selectively arsenopyrite is oxidized and after its complete oxidation, pyrite oxidation is started (Chandraprabha *et al* 2002). Fernandez *et al.* (1995) deciphered the stepwise mechanism for arsenopyrite oxidation. The first step is characterized by a slow but strong adhesion of bacteria to mineral surfaces, the appearance of sulphur surface and weak solubilization of Fe^{+2}, As^{+3} and As^{+5}.

In second step, growth of unattached bacteria begins and they solubilize Fe^{2+} in solution. This solubilized ferrous iron (Fe^{+2}) is oxidized to ferric iron (Fe^{+3}). This generated ferric iron solubilizes the metal sulfide directly, get reduced to Fe^{+2} and is regenerated by bacterial oxidation.

At this time, bioleaching cycle takes place and coarse surface phase of ferric arsenate takes place, in which deep ovoid pores on mineral surfaces appear. At the end of bioleaching cycle, the high concentration of Fe^{+3} and As^{+5} in solution promotes the precipitation of amorphous ferric arsenate in the leachate (Fernandez *et al.*, 1995).

It was found out that attachment of bacteria is faster on pyrite than arsenopyrite and number of bacteria attaching on pyrite is also higher than arsenopyrite (Chandraprabha *et al* 2003). The oxidative step of arsenopyrite is given by following equation (Lindstrom *et al*, 1992; Chandraprabha *et al.* 2002):

$$4FeAsS + 13O_2 + 6H_2O \rightarrow 4H_3AsO_4 + 4FeSO_4 \quad \text{.....(5)}$$

$$2FeAsS + Fe_2(SO_4)_3 + 6O_2 + 4H_2O \rightarrow 2H_3AsO_4 + 4FeSO_4 + H_2SO_4 \quad \text{.....(6)}$$

$$2\ H_3AsO_4 + Fe_2(SO_4)_3 \rightarrow 2FeAsO_4 + 3H_2SO_4 \quad \text{.. .....(7)}$$

Here bacterial oxidation takes place in equation 5 only. Fe^{+2} is oxidized to Fe^{+3} by bacteria at low pH and by equation 7, As^{+5} is transferred to $FeAsO_4$, which precipitates. However, arsenopyrite oxidation also leads to the dissolution of arsenic in the form of As^{+3}, which is described as below:

$2FeAsS + 5.5O_2 + 3H_2O \rightarrow 2H_3AsO_3 + 2FeSO_4$.....(8)

$2FeAsS + 6.5O_2 + 3H_2O \rightarrow 2H_3AsO_4 + 2FeSO_4$.....(9)

It was supposed that As^{+3} is oxidized directly to As^{+5} by oxygen and therefore $FeAsO_4$ is formed and arsenic is removed from the solution, but it was found out that in the normal conditions As^{+3} is not oxidizable by oxygen, but ozone (O_3) is needed for this oxidation. Hence it is possible that As^{+3} is oxidized to As^{+5} by Fe^{+3}.

The overall reaction can be given by (Agate, 1982; Rawling and Silver 1995):

$$FeAsS + 3½\ O_2 + H_2O \xrightarrow{\text{microorganisms}} FeAsO_4 + H_2SO_4 \quad \text{.....(10)}$$

6.1.3 FACTORS AFFECTING THE METAL LEACHING BACTERIA

1. **Metal tolerance**: Although *A. ferrooxidans* has a notably high tolerance for heavy metals, presence of very high concentration of metals in leach liquor may decrease the rate of metal leaching or sometimes inhibit the growth of the leaching organisms completely.

2. **Redox potential and pH**: Maximum leaching by *A. ferrooxidans* is achieved at pH 1.75 to 2.5 (Lazaroff *et al.*, 1982; Olson, 1991). At pH above 2.5, precipitation of jarosites takes place on the surface of sulfide minerals impeding their oxidation, which can be minimized by maintaining reactor conditions at extremely low pH by adding HCl or H_2SO_4, and keeping low redox potentials (Lazaroff *et al.*, 1982; Menon *et al.*, 1996; Yahya and Johnson,

2002). At a pH lesser or more than optimal pH of organism, generation time of cell also increases resulting in slower growth (Mykytczuk *et al.*, 2010). Once growth has initiated, even if pH increases to 3.5, it will not inhibit cell development or cell function (Olson 1991; Lindstrom *et al.*, 1992; Schroter and Sand, 1992; Bhatti *et al.*, 1993).

During the oxidation of ferrous iron by *A. ferrooxidans,* the Eh is expected to increase upto +747 mV in ferric-ferrous system. However, in reality this value is rarely approached, because oxidized iron precipitates as jarosite and lower down Fe^{+3}/Fe^{+2} ratio. Hence, during ferrous oxidation Eh values of +190 to +550 mV are seen, while for metal sulfides, it is between +220 to +550 mV. Cordoba *et al.* (2008) reported that increase in the redox potential upto 600 mV increased copper dissolution in the presence of silver ions.

3. **Nutrients**: In most of the media recommended for iron oxidizing *Acidithiobacilli*, excess concentration of all required salts are added including ammonium sulphate, phosphate, magnesium and calcium. Ferrous sulphate is the main energy source for *A. ferrooxidans* which can be replaced by minerals containing ferrous iron or sulphur.

   Ammonium salts and probably nitrate can be used as nitrogen source. However *A. ferrooxidans* grows better on nitrogen obtained from NH_3 than NO_3 (Tuovinen *et al.*, 1971). Some heterotroph may assist in nitrogen fixation, but they would also interfere with leaching by physical blocking of sulfide surfaces and leach flows. However, in presence of an acid and metal tolerant, non-symbiotic nitrogen fixer *Beijerinckia lacticogens*, *A. ferrooxidans* gave five fold improvements in the leaching, which shows that fixing of atmospheric nitrogen by another heterotroph may also satisfy nitrogen requirement of leaching *Acidithiobacilli.*

4. **Water potential**: It is found that *A. ferrooxidans* can grow and oxidize iron at water potentials of –1.5 X 10^6 Pa to –2 X 10^6 Pa. Environments which have too low water potential can be adjusted using sodium chloride and glycerol at a concentration which are not inhibitory to it.

5. **Surface tension**: Addition of surface-active agents, such as tweens have adverse effect on metal leaching. The isoelectric point of *A. ferrooxidans* is at pH 4.43. During leaching at pH 2 or 2.5, the surface of bacterium is positively charged. If surface active agents are added, they reverse the charge and make the organism hydrophobic. This change in wettability is responsible for adverse effect on leaching.

6. **Dissolved gases**: Boon and Heijnen (1998) found out that limitation of gas-liquid CO_2 transfer and exhaustion of CO_2 in the gas phase occurs very often due to increased pulp density and it causes the decrease in the bacterial oxidation rate constant at increasing slurry densities (Boon and Heijnen, 1998; Mousavi *et al.*, 2006).

   Oxygen is main oxidant in *A. ferrooxidans* and to overcome the limitation of air and CO_2 transfer, various aeration and mixing devices have been reported (Tipre *et al.*, 2001). Foucher *et al.* (2003) used a suspended solid bubble column over classical mechanically agitated bioreactor to reduce operating cost and to decrease the stress induced on the bacteria by the propellers (Acevedo and Gentina, 1989; Acevedo *et al.*, 1998; Foucher *et al* 2003). To maximize oxygen transfer in ore, various method of aeration like percolation, trickle and flood leaching have been studied (Puhakka and Tuovinen, 1986a, 1986b; Menon and Dave, 1995).

7. **Temperature**: Thermophiles at higher temperature (eg. Thermophilic archean *Sulfolobus* sp. at 68°C) solubilize greater

amount of metal as compared to mesophiles (Norris *et al.*, 1980; Lindstrom *et al*, 1992; Munoz *et al* 2006). Since thermophilic bacteria such as *Sulfobacillus, Acidianus*, and *Sulfolobus* spp. show higher bioleaching rates, they are now being used in mixed cultures with *A. ferrooxidans* for commercial dump and heap leaching processes for metal recovery (Mousavi *et al.*, 2006).

The growth of *A. ferrooxidans* is reported in the temperature range of 4ºC - 37ºC. Eventhough upper temperature limit for growth of *A. ferrooxidans* is 40ºC – 45ºC, its ferrous oxidizing activity is inhibited at temperature higher than 35 ºC (Modak *et al.*, 1996; Mishra *et al.*, 2008). Ahonen and Tuovinen (1992) found 28ºC temperature to be optimum for sphalerite, pyrrhotite and pentalandite bioleaching. Moderately thermophilic mixed culture oxidized arsenopyrite faster at 45°C than *A. ferrooxidans* at 22°C (Duarte *et al.*, 1993; Tuovinen *et al*, 1994).

8. **Light**: Visible, IR and UV light have inhibitory effect on some acidithiobacilli, with blue end of the spectrum showing the highest inhibition. Presence of particulate matter and ferric iron offers some protection from visible rays.

9. **Pressure**: *In situ* leaching of chalcopyrite under simulated, deep solution mining has an oxygen pressure of 2.7 X 10^5 Pa to 1.1 X 10^7Pa, which becomes an important factor during *in situ* leaching. Chemoautotrophic bacteria are barotolerant and can withstand hydrostatic pressures of 1.5 x 10^7 Pa.

10. **Particle size**: Greater yield can be achieved if the specific surface area of the ore is increased by reducing particle size (Dave *et al.*, 1979). Below a critical size it can cause increase in gangue and results in a dilution of substrate (Dave and Mathur, 1987; Nemati *et al.*, 2000).

11. **Substrate concentration**: The concentration of solid substrate is referred as pulp density or solid: liquid ratio. Increasing the available substrate results in increase of absolute quantity of metal values solubilized. But these accumulated metals in leaching medium may become toxic for leaching bacteria and adversely affect the leaching process (Tipre *et al.*, 1998). The substrate concentration is increased gradually to adapt the cell to higher concentration of metals and higher oxidation of ore is achieved in many cases (Attia, 1985; Murthy and Natarajan, 1992; Tupikina *et al.*, 2005).

    However when solid: liquid ratio is high, the leaching environment becomes complex and extraction rates may decrease because solid interferes with the mass transfer of O_2 and CO_2 gases to microorganisms (Dave, 1980; Roy-Mahapatra *et al.*, 1985; Dave and Mathur, 1987; Dave *et al.*, 2001).

12. **Minerology**: ores are classified on the basis of their resistance to leaching since:

- Some ores are recalcitrant to dissolution (eg chalcopyrite)
- Some ores after dissolution release toxic products, which is harmful to leaching organisms.
- Ores may release insoluble product resistant to bacterial attack.

    It is found that in multimetallic sulfides, the metals with a low solubility product have the fastest metal extraction rate. Hence ore mineralogy affects leaching. Gangue mineralogy might be useful in deciding buffering capacity, adsorption of cation/anion and affecting water potential.

6.2 MATERIALS AND METHODS

6.2.1 MEDIA OPTIMIZATION FOR PYRITE OXIDATION

Commercially available pyrite (FeS_2) (Astron, India) was used for all pyrite biooxidation studies. Pyrite granules were finely ground and sieved. Pyrite crushed to -200+325 mesh size fraction was used.

For obtaining higher pyrite oxidation by different isolates, inorganic and organic media were compared. 9K medium modified with respect to 2% pyrite addition instead of reported 4.4% ferrous sulphate, was used as inorganic medium. The organic medium was prepared by addition of 0.025% tripticase soy supplement (TS) in above medium. A working volume of 100 ml in 250 ml erlenmeyer flask was set with the above medium. Pyrite was sterilized by exposure to 256 nm UV light at 15 cm distance for 10 min. and added in all the flasks at 2% concentration. Medium pH was adjusted to 1.9 by addition of 10% H_2SO_4 after addition of TS. Medium was inoculated with 10% v/v of actively growing cultures of SRD 5, HGM 26, HGM 30 and HGM 38. After inoculation, the flasks had cell load of 1.2 – 1.5 X 10^7 cells/ml. The flasks were incubated at environmental shaker at 30±2°C at 150 rpm. Ferrous released in the medium from pyrite was estimated every 24 h intervals.

6.2.2 PYRITE OXIDATION KINETIC STUDY

Pyrite un-adapted isolates were used for the kinetic study of pyrite oxidation. The inoculum was prepared from cultures pre-grown in 9K medium containing 40 g/l ferrous sulfate. Culture showing >95% oxidation was harvested by method shown in appendix II and pellet obtained was washed twice with sterile pH 1.8 distilled water. Finally the washed pellet was resuspended in sterile distilled water to prepare inoculum having cell load of 10^8 cells/ml. Oxidation of pyrite was measured by inoculating 10% v/v inoculum into a set of flasks having 100ml 9k basal medium and 0.25% to 4% w/v pyrite.

The inoculated flasks were incubated at 30±2°C on environmental shaker at 150 rpm for 60 days. Samples were removed at regular intervals and dissolved ferrous concentration, pH and soluble total iron were analyzed as given in appendix II.

6.2.3 ARSENOPYRITE OXIDATION KINETIC STUDY

After adaptation on arsenic and technical grade pyrite, isolate SRD 5 was studied for arsenopyrite oxidation. Arsenopyrite ore sample was procured from Hutti gold mine ltd., Raichur district, Karnataka. Ore sample was crushed and finely ground. Different size fractions were separated by sieving and –200+325# mesh size fraction was used in the study. Chemical composition of arsenopyrite ore was studied by EDAX analysis. For arsenopyrite oxidation kinetic study, 0.1g to 8g ore was weighed and sterilized separately by exposure to 256 nm UV light at 15 cm distance for 10 min. before adding in 100 ml sterile 9K medium in 250 ml erlenmeyer flasks giving 0.1% to 8% P.D. Culture of SRD 5 was harvested and inoculum was made having cell load of 5 x 10^8 cells/ml. The arsenopyrite containing flasks were inoculated with 10% v/v individual inoculum and incubated on environmental shaker at 30±2°C temperature at 150 rpm. Samples were withdrawn aseptically from each flask at every 24 hour interval and dissolved ferrous and total iron were measured.

6.3 RESULT AND DISCUSSION

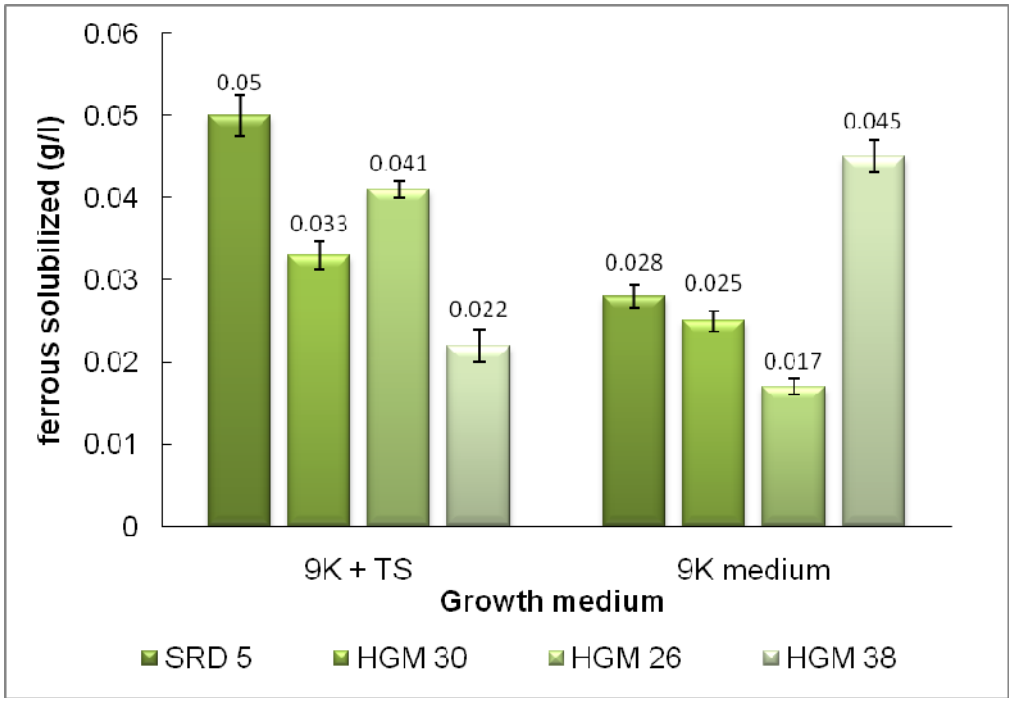
6.3.1 MEDIA OPTIMIZATION FOR PYRITE OXIDATION

Medium was optimized for pyrite oxidation by 4 isolates SRD 5, HGM 26, HGM 30 and HGM 38. Graph 6.1 shows the amount of ferrous leached from pyrite by these isolates in presence and absence of organic supplement. As shown in graph 6.1, SRD 5 showed 0.05 g/L soluble ferrous in TS containing 9K medium at the end of 11 days as compared to 0.028 in 9K medium alone. Similarly HGM 30 and HGM 26 showed 0.033 g/L and 0.041 g/L soluble ferrous respectively in TS containing medium, against 0.025 g/L and 0.017 g/L respectively in

9K medium alone. Hence, it can be summarized that isolates SRD 5, HGM 30 and HGM 26 showed better pyrite oxidation in presence of organic matter in medium. In case of HGM 38, better solubilization of pyrite (0.045 g/L) was observed in inorganic 9K medium as compared to 0.022 g/L in presence of TS (organic medium).

To confirm this pattern, HGM 38 was grown in two different inorganic medium and 1 organic medium. 9K and DBJ basal medium (Johnson *et al.* 1987, Johnson and McGinness 1991) without tripticase soy supplement were used as inorganic medium, while DBJ medium with tripticase soy supplement was used as organic medium. In this case also HGM 38 showed good response to inorganic 9K and DBJ basal medium, however less ferrous was solubilized when TS was added in DBJ basal medium.

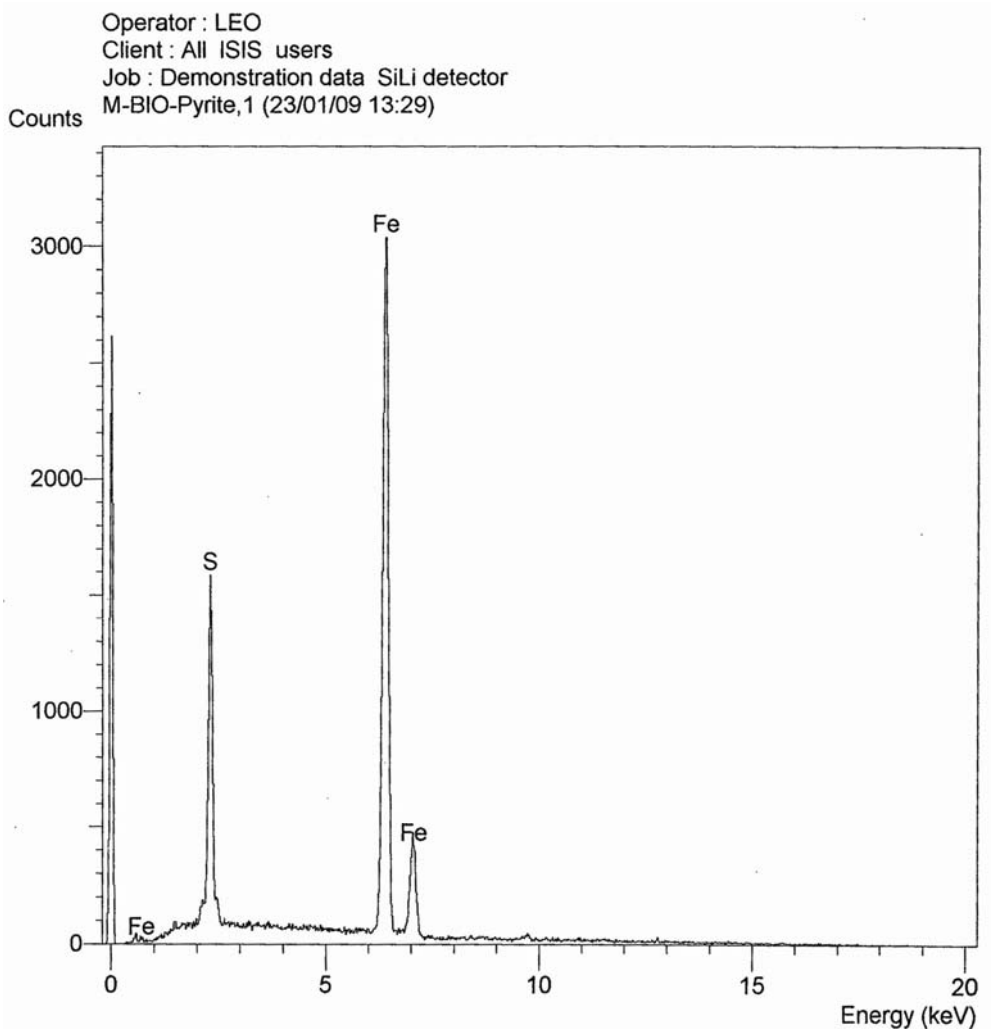
Graph 6.1 : Ferrous solubilized from pyrite in 9K and 9K+TS supplement medium



6.3.2 PYRITE OXIDATION KINETIC STUDY

The biooxidation of commercially available pyrite (FeS_2) (Astron, India) by autotrophic isolates HGM 26, HGM 30, HGM 38 and SRD 5 was studied. Before starting the biooxidation of pyrite, the total amount of iron present in it was measured by EDAX analysis and confirmed by chemical digestion of ore and its total iron analysis. The composition of pyrite as obtained by EDAX analysis is shown in figure 6.1

Figure 6.1: Composition of pyrite as obtained by EDAX analysis



Element	Content (%)
Iron (Fe)	80.12
Sulphur (S)	19.82
Total	100.00

Since the iron content in commercially available pyrite was 80.12%, amount of total iron present in each flask of 0.05% to 4% pyrite was calculated. The actual iron content of all the flasks as obtained by EDAX as well as colourimetric analysis is shown in table 4.1. These iron concentrations present in each flasks were used as 100% concentration to compare extent of iron solubilized by pyrite biooxidation. The extent of leaching was monitored during entire course of pyrite biooxidation by measuring ferrous iron (Fe^{+2}) and total iron (combined amount of ferrous Fe^{+2} and ferric Fe^{+3} iron) present in the leachate.

Table 6.1 : Actual iron content present in ore

Pyrite conc. (%)	Iron content (g/L)	
	EDAX analysis	**Colourimetric estimation**
0.05	0.40	0.40
0.1	0.80	0.80
0.25	2.00	2.02
0.5	4.01	4.05
1	8.01	8.46
2	16.02	16.41
4	32.05	32.32

6.3.2.1 SOLUBILIZATION OF FERROUS IRON AND TOTAL IRON

As the pulp density of pyrite increased from 0.05% to 4% in the medium; the amount of solubilized ferrous iron from pyrite also increased for all the isolates. In an interlaboratory comparison for oxidation of 1% pyrite, average pyrite oxidation rate of 12.4 mg/l/h was reported by Olson (1991).

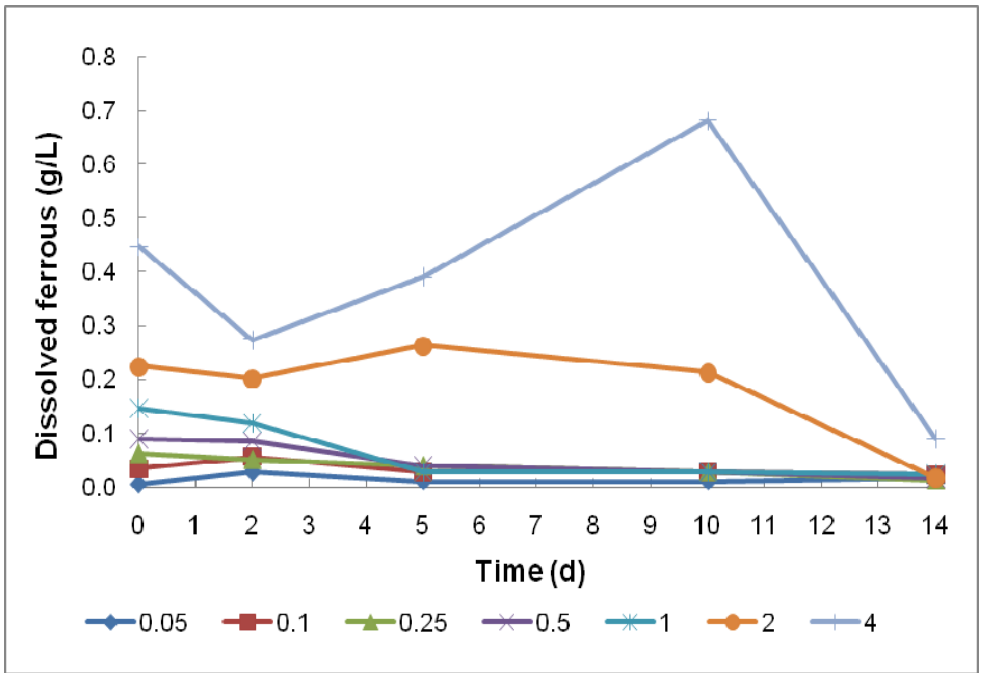
During pyrite oxidation by isolate SRD 5; amount of solubilized ferrous iron remained stable in 0.05 % to 1% P.D., instead decrease in ferrous was observed, which went below measurable amount after 4th day (graph 6.2). However the total iron in leachate solution increased

continuously. Therefore it can be understood that rate of ferrous iron oxidation to ferric iron was higher as compared to ferrous iron solubilization from pyrite. Therefore amount of soluble ferrous iron did not increase in the leachate. However very little increase from 0.2 g/l to 0.3 g/l was observed in 2% pyrite flask during 2nd to 5th day. Fifth day onward the amount of ferrous remained stable and gradually deceased after 10th day. In case of 4% pyrite, initial ferrous was measured at 0.45 g/l on day of inoculation, which decreased in 2 days possibly because of rapid oxidation of ferrous by organisms. After 2 days amount of solubilized ferrous iron increased upto 0.7 g/l in 10 days, after which no further increase was observed.

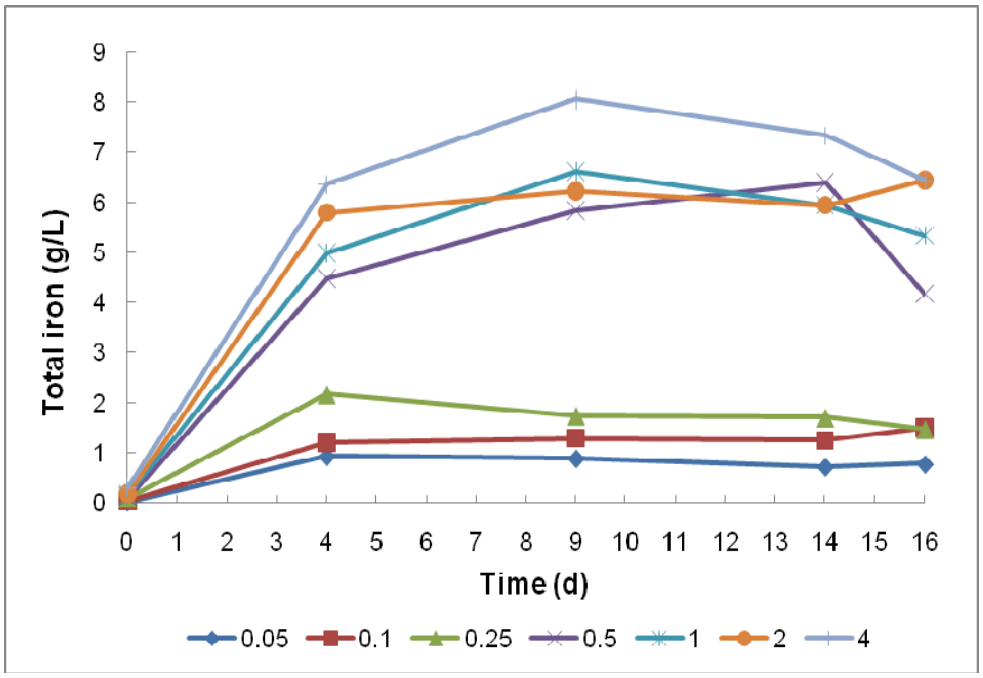
As shown in graph 6.3, total iron content increased rapidly during initial 4 days. During this time, amount of dissolved ferrous decreased. Both these observation indicate rapid oxidation without any lag period required. Within 4 days, >99% leaching was over in flasks having 0.05%, 0.1% and 0.5% pyrite, but in 0.25% pyrite containing flask only 87% ferrous solubilization was observed. However, within this time, 1%, 2% and 4% pyrite was also oxidized in a fair extent, where 59%, 35% and 20% oxidation was over. In 1% pyrite containing flask, 78% oxidation was observed in 16 days. As against this, in 2% and 4% pyrite containing flasks, only 39% and 25% biooxidation could be obtained respectively.

As can be seen from graph 6.4, rate of pyrite oxidation (POR) at lower concentrations of pyrite i.e. 0.05%, 0.1%, 0.25% and 0.5% increased sharply as compared to pyrite oxidation rate at higher pulp densities i.e. 2% and 4%. On 9th day of incubation, 0.23, 0.29, 0.52 and 1.09 $g.l^{-1}.d^{-1}$ POR was noted in 0.05%, 0.1%, 0.25% and 0.5% pyrite containing flasks respectively. At 1%, 2% and 4% pulp densities the pyrite oxidation rate was 1.2, 1.4 and 1.51 $g.l^{-1}.d^{-1}$ respectively. Reciprocal plot of pyrite oxidation rate (1/V) vs reciprocal of pulp density (1/S %) (graph 6.5) gave 0.26 K_m value and V_{max} was 1320 $mg.l^{-1}.d^{-1}$ or 55 $mg.l^{-1}.h^{-1}$.

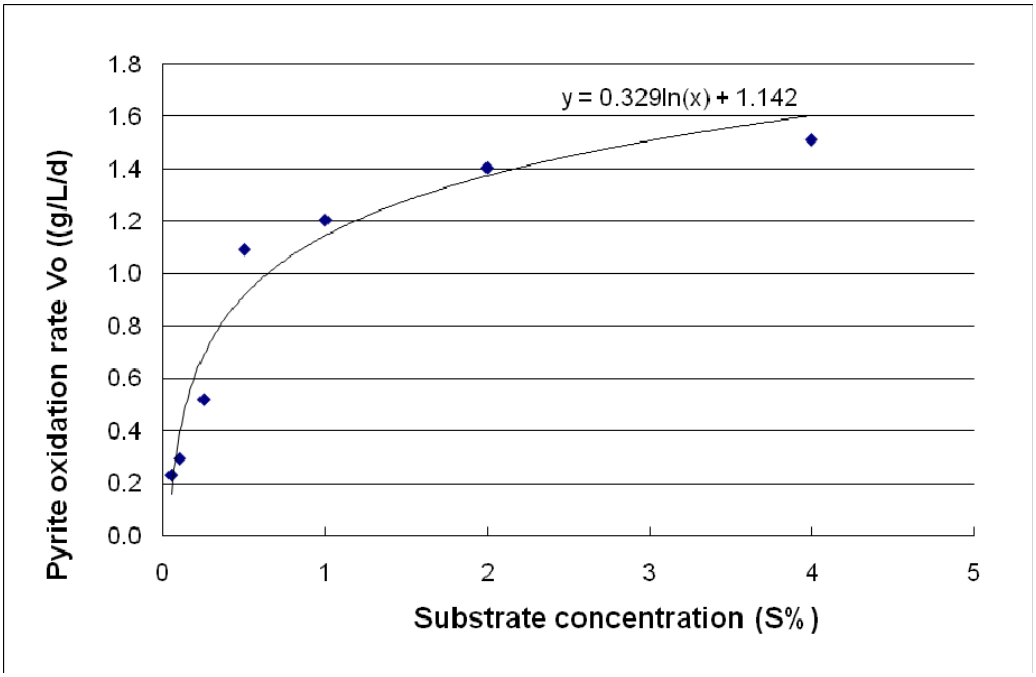
Graph 6.2: Ferrous solubilization from pyrite by isolate SRD 5



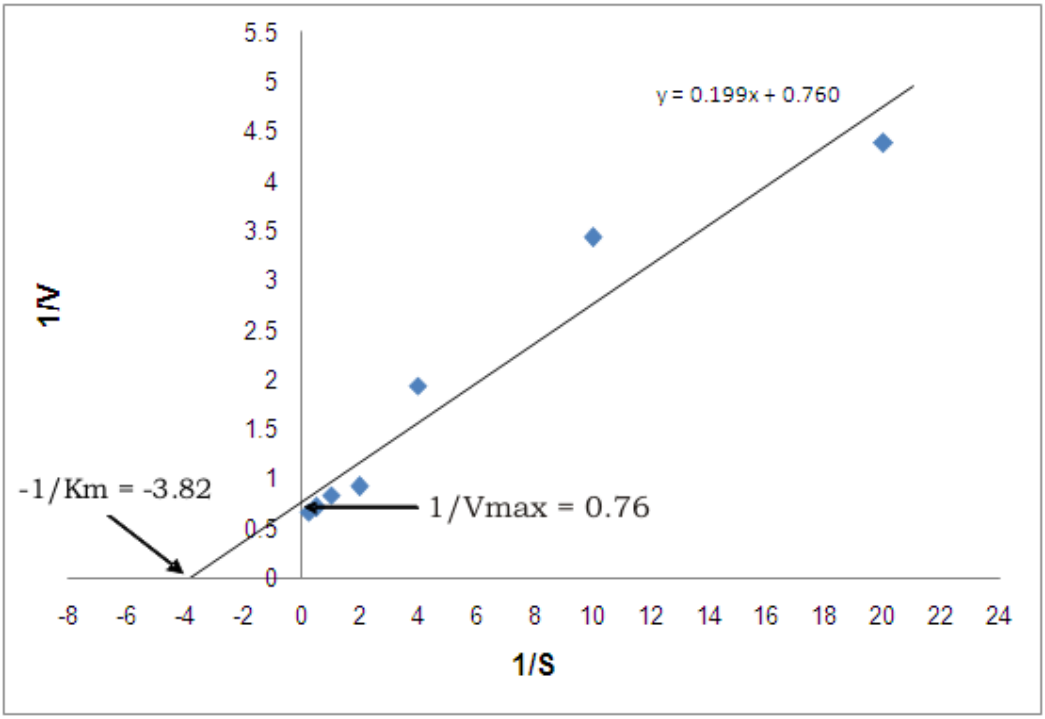
Graph 6.3: Dissolved total iron from pyrite by isolate SRD 5



Graph 6.4 : Rate of pyrite biooxidation by isolate SRD 5



Graph 6.5 : Reciprocal of Pyrite oxidation rate (1/Vo) vs reciprocal of pulp density (1/S%) – SRD 5



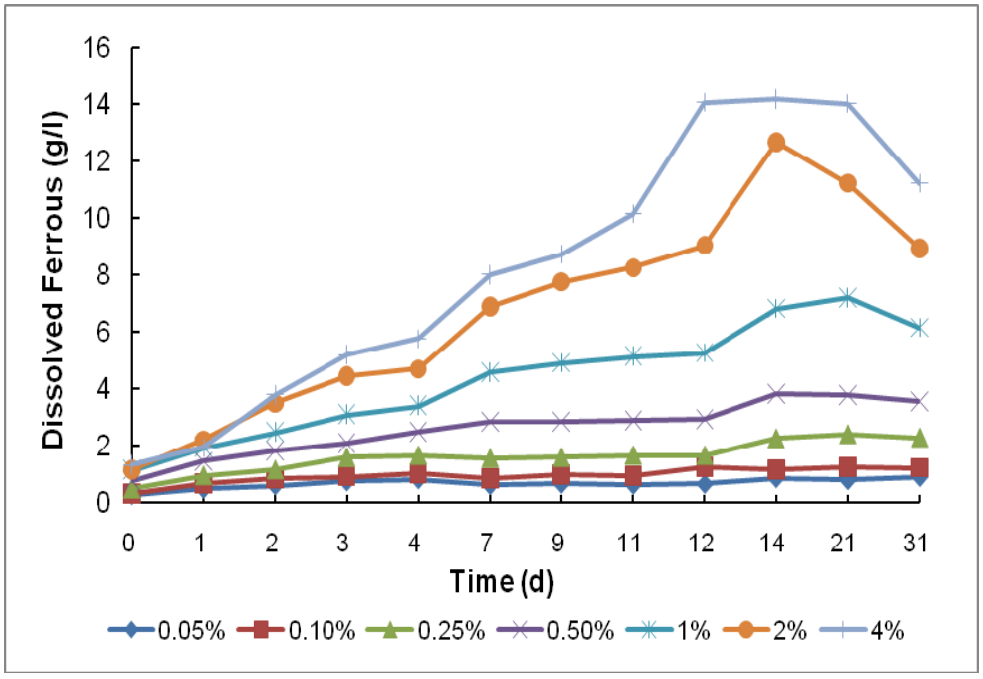
$1/V_{max}$ =	0.76	;	V_{max} = 1320 mg/l/d
-1/Km =	- 3.82	;	Km = 2.6×10^{-1}

As shown in graph 6.6, in case of HGM 26, the maximum ferrous iron was solubilized from pyrite in 14 days time, which decreased afterwards due to its continuous oxidation to ferric iron. In 0.05, 1 and 0.25% pulp densities of pyrite, 0.85, 1.18 and 2.26 g/l ferrous iron was solubilized within 14 days. This indicated that >95% leaching of pyrite was obtained in upto 0.25% P.D. In 0.5% P.D., 3.85 g/l ferrous was present in leachate on 14th day against 4.48 g/l ferrous available in pyrite, which correspond to 86% leaching.

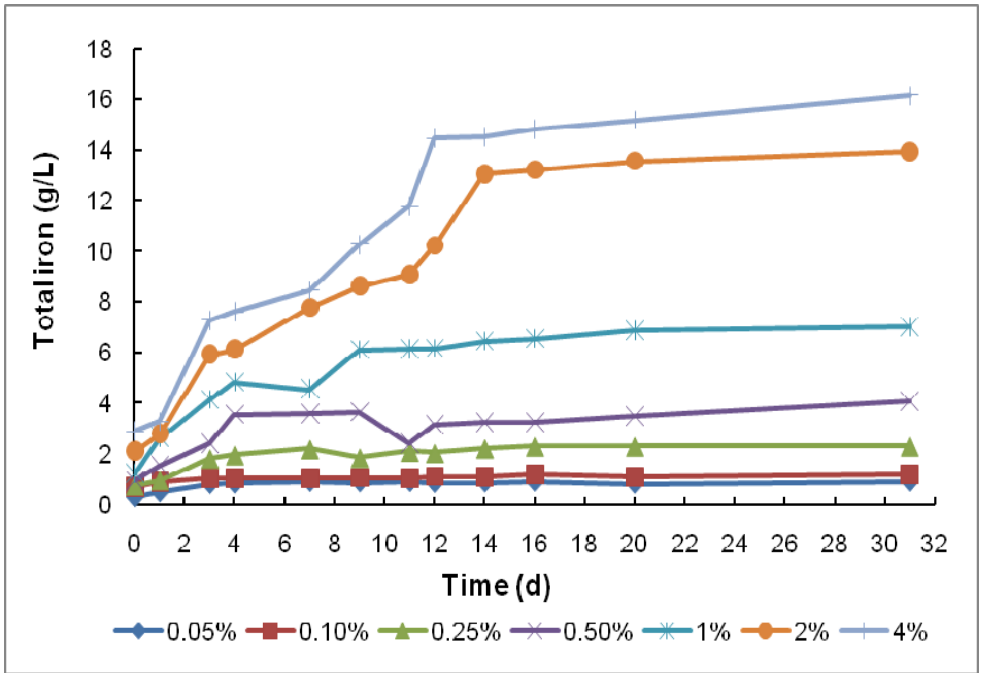
On 14th day, 6.81 g/l ferrous was measured in 1% P.D. flask as against 8.46 g/l total iron present in pyrite, which corresponded to 80% leaching. In the similar way, from 2% and 4% pulp densities 12.68 g/l and 14.2 g/l ferrous was measured in the leachate from 16.41 and 32.3g/l total ferrous available in pyrite. This accounts to 77.3% and 44% leaching. Hence looking to the overall data it is observed that in higher pulp densities viz. 0.5, 1, 2 and 4%, the percent ferrous leaching obtained was 86, 80, 77 and 44% respectively and therefore as pulp density was increased from 0.5 to 4%, lower leaching of iron was observed.

As shown in total iron analysis in graph 6.7, the rate of pyrite oxidation was low during initial 24h, which increased sharply after 24 h. This lag phase in pyrite oxidation may be due to adaptation of iron grown cells to pyrite leaching environment. Out of 0.9 g/l total iron content present in 0.05% pyrite, 0.81 g/l was leached within initial 3 days, which corresponded to 90% leaching. Similarly in 0.1% and 0.25% P.D., pyrite oxidation was over in 3 d and 7 d respectively. This accounted to 1.04 and 2.19 g/l total iron in medium as compared to 1.30 and 2.49 g/l total iron present respectively in flasks. This corresponded to 80 and 88% leaching respectively. In 2% pyrite containing flask, maximum oxidation had completed in 14 days which was 13.05 g/l out of total 16.41 g/l, i.e 79% leaching. Similarly in 4% P.D., 14.53 g/l iron was leached out of total 32.32 g/l, which accounted for 45% leaching.

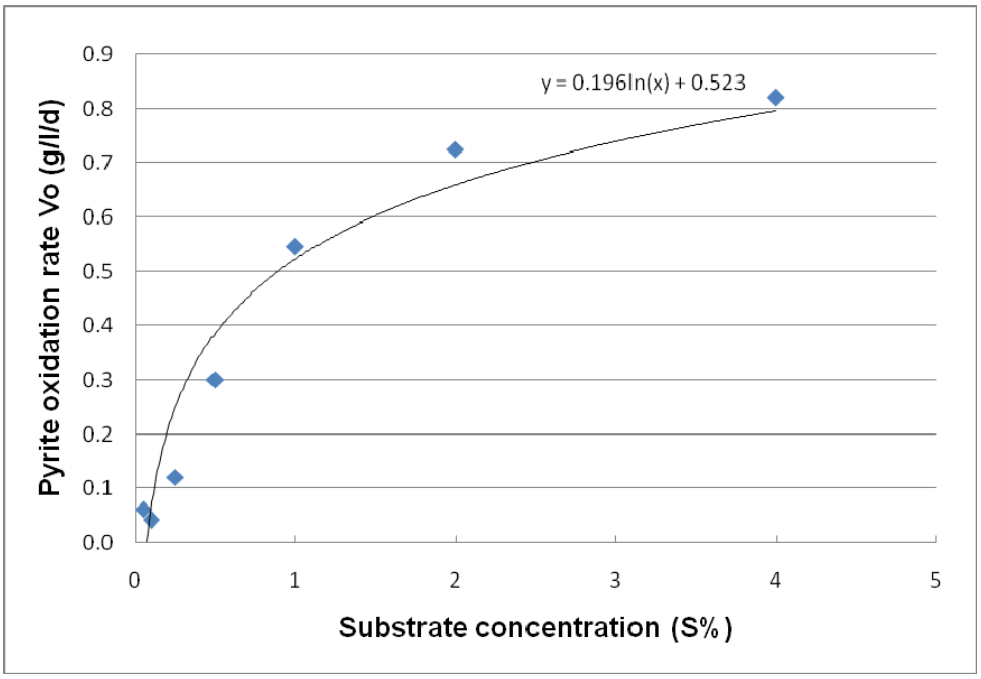
Graph 6.6: Ferrous solubilization from pyrite by isolate HGM 26



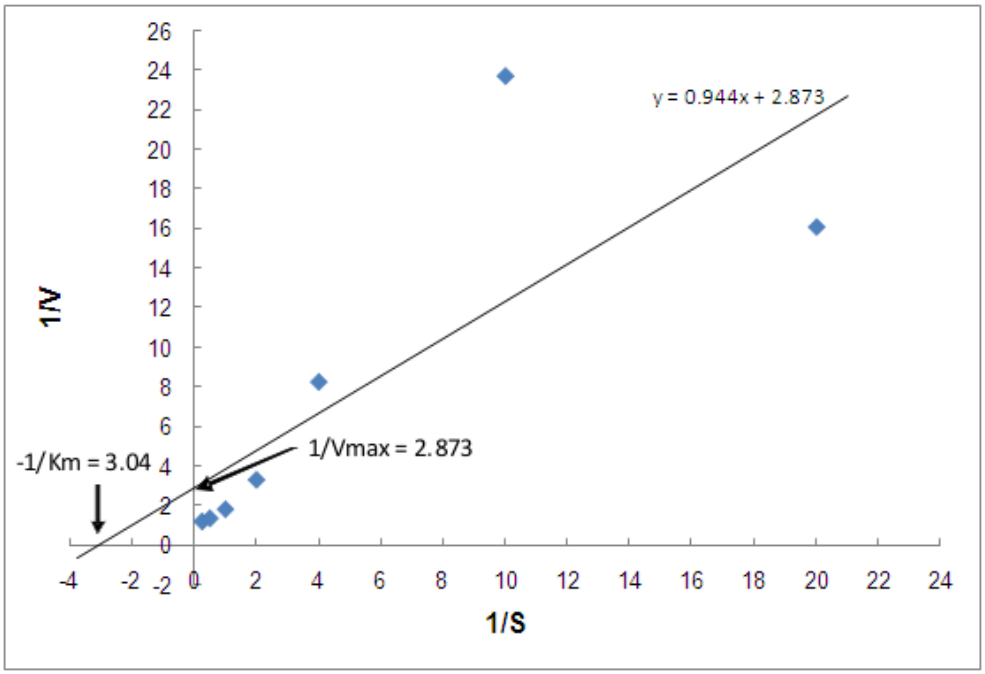
Graph 6.7: Dissolved total iron from pyrite by isolate HGM 26



Graph 6.8 : Rate of pyrite biooxidation by isolate HGM 26



Graph 6.9 : Reciprocal of Pyrite oxidation rate (1/Vo) vs reciprocal of pulp density (1/S%) – HGM 26



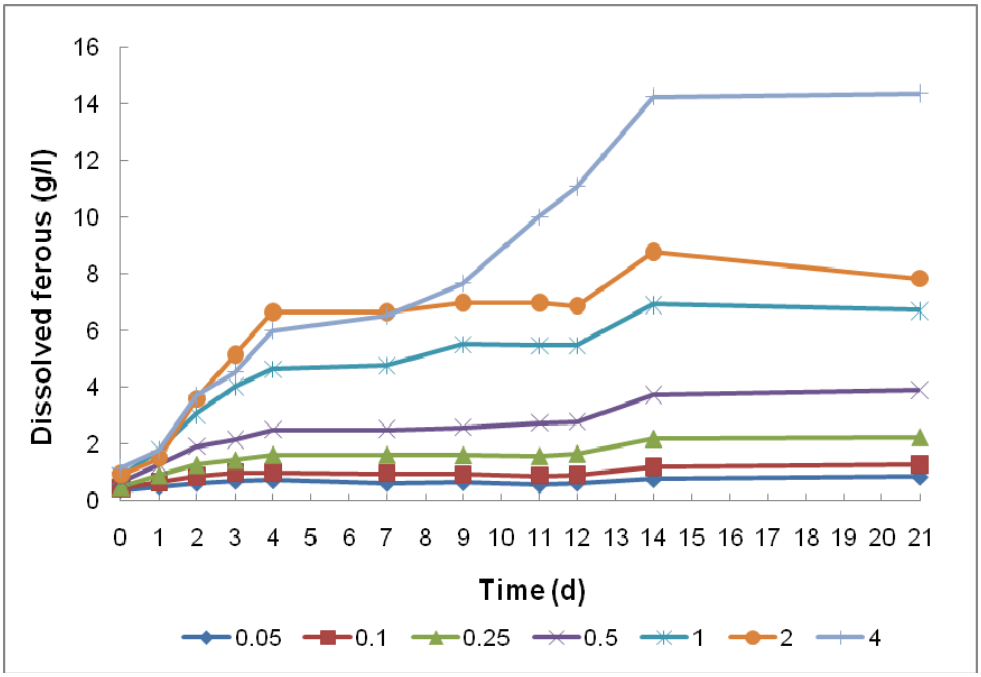
$1/V_{max} = 2.873$	;	$V_{max} = 350$ mg/l/d
$-1/Km = -3.04$	;	$Km = 3.3 \times 10^{-1}$

In HGM 30, the ferrous iron content showed rather steep increase during initial 4 days, which was more clearly visible in 1, 2 and 4% pulp densities and the soluble ferrous iron content of 4.63, 6.63 and 5.99 g/l was observed. In 4 days itself, 54.7%, 40% and 18.5% leaching was completed in 1, 2 and 4% pyrite containing flasks (graph 6.10). However, after 4 days, slow increase was observed in 1 and 2% flasks till 14 days. Till then maximum ferrous was leached out and after which there was no increase in ferrous iron concentration except 4% P.D., in which solubilized ferrous iron content increased comparatively faster than 1% & 2% P.D and reached upto 14.35 g/l ferrous iron. As a result, >80% oxidation was observed in 0.05% to 1% pulp densities. In 2% and 4% pulp densities, only 53% and 44% oxidation was obtained.

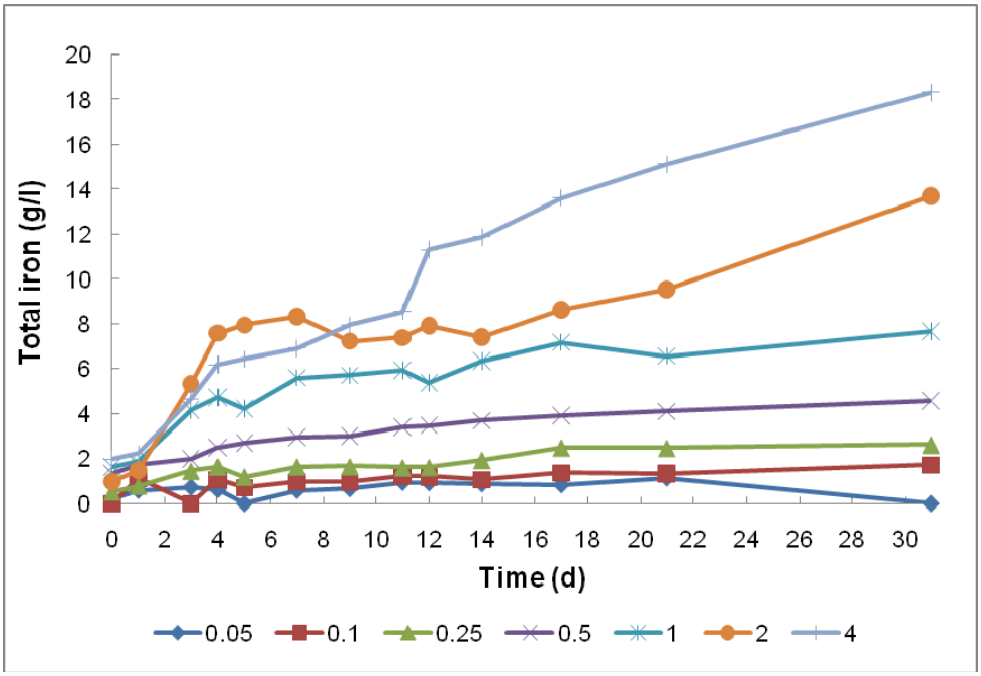
As shown in graph 6.11, when total iron content from pyrite oxidation flask was observed, more than 92% oxidation was achieved in pulp densities 0.05% to 0.5%. In pulp densities 1%, 2% and 4%, 77.8%, 58% and 46.8% oxidation was observed. However the amount of ferrous iron solubilized from 2% pyrite was highest among all pulp densities during initial 7 days. Moreover, during analysis of total iron content, highest oxidation was also observed from 2% pyrite in same time period. This indicated that isolate HGM 30 required 7 days time for adaptation on 4% pyrite. From 8^{th} day onward highest amount of ferrous iron was solubilized from 4% pyrite. This also resulted into highest amount of ferric in medium.

Rate of pyrite oxidation by HGM 30 is plotted against substrate concentration as shown in graph 6.12. When reciprocal of pyrite oxidation rate (1/Vo) was plotted against reciprocal of pulp density (1/S %) as depicted in graph 6.13, a K_m value of 0.35 was obtained. Moreover V_{max} for pyrite oxidation by isolate HGM 30 was found to be 870 mg/l/d.

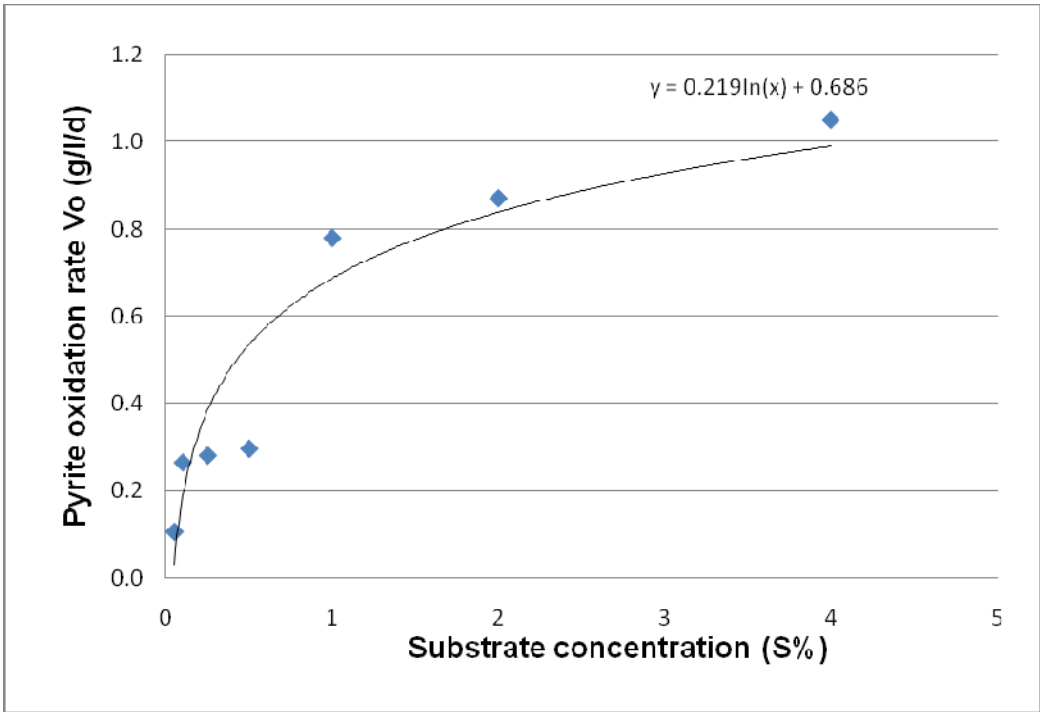
Graph 6.10: Ferrous solubilization from pyrite by isolate HGM 30



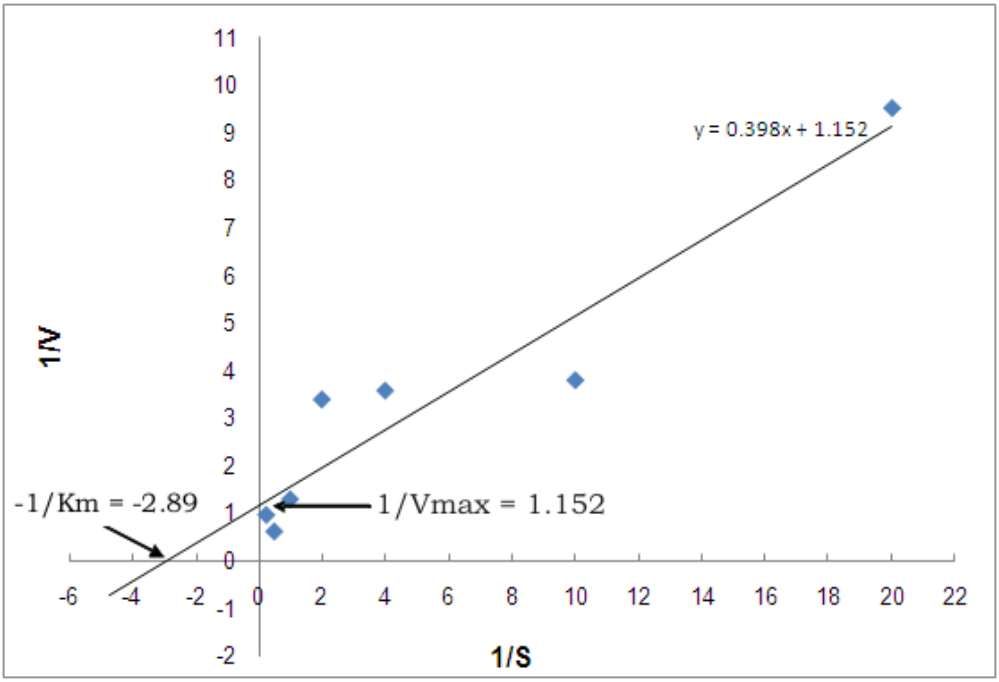
Graph 6.11: Dissolved total iron from pyrite by isolate HGM 30



Graph 6.12 : Rate of pyrite biooxidation by isolate HGM 30



Graph 6.13 : Reciprocal of Pyrite oxidation rate (1/Vo) vs reciprocal of pulp density (1/S%) – HGM 30



$1/V_{max}$ =	1.152	;	V_{max} = 870 mg/l/d
-1/Km =	- 2.89	;	Km = 3.5×10^{-1}

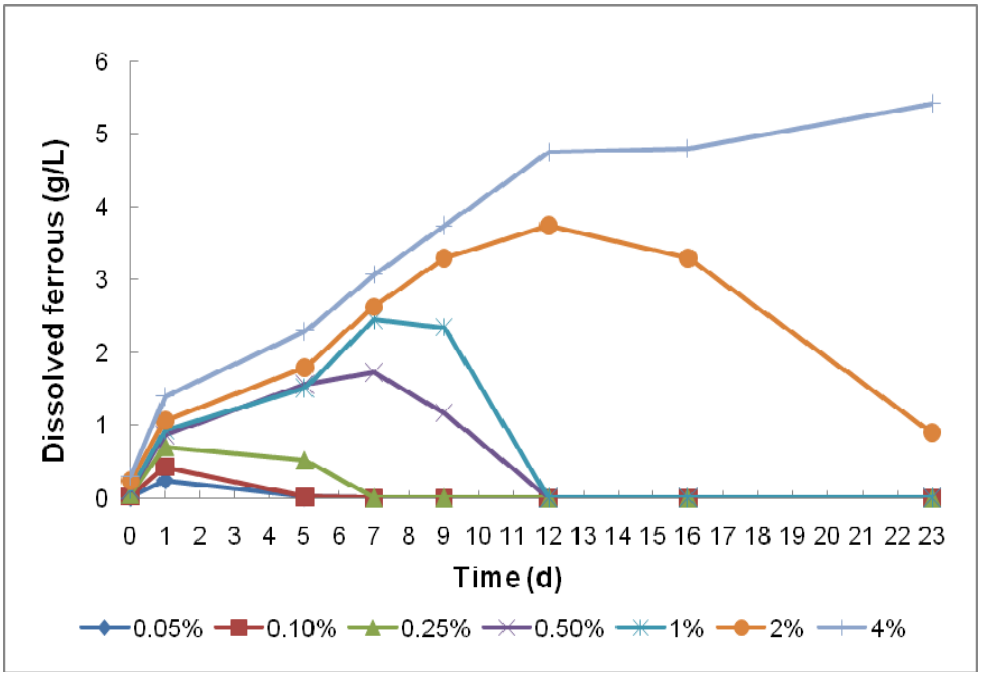
In HGM 38, increase in amount of soluble ferrous was observed at all concentrations of pyrite (graph 6.14). During initial 24h, ferrous iron content in all the flasks increased at a faster rate. This indicated that isolate HGM 38 started biooxidation in all the concentration of pyrite viz. 0.05% to 4% P.D., without any lag period. However after 24h of incubation, decrease in the amount of solubilized ferrous iron in 0.05%, 0.1% and 0.25% P.D. flasks was observed.

If the rate of soluble ferrous biooxidation in leachate is higher than ferrous solubilization from pyrite, it will result in the observed decrease in amount of Fe^{+2} in pyrite leaching flasks. From 6^{th} day onward, the ferrous solubilization rate showed sharp increase and along with that total iron content of the flask also increased rapidly (Graph 6.15). At 0.5% and 1% P.D., the amount of soluble ferrous iron increased upto 1.7 and 2.45 g/l on 7^{th} day. After that no increase in soluble ferrous iron was observed. In case of 2% P.D., ferrous iron was continuously leached and maximum ferrous iron was observed on 12^{th} day which was 3.7 g/l, after that gradual decrease was observed, while in case of 4% pyrite continuous increase in soluble ferrous iron was seen upto 23 days.

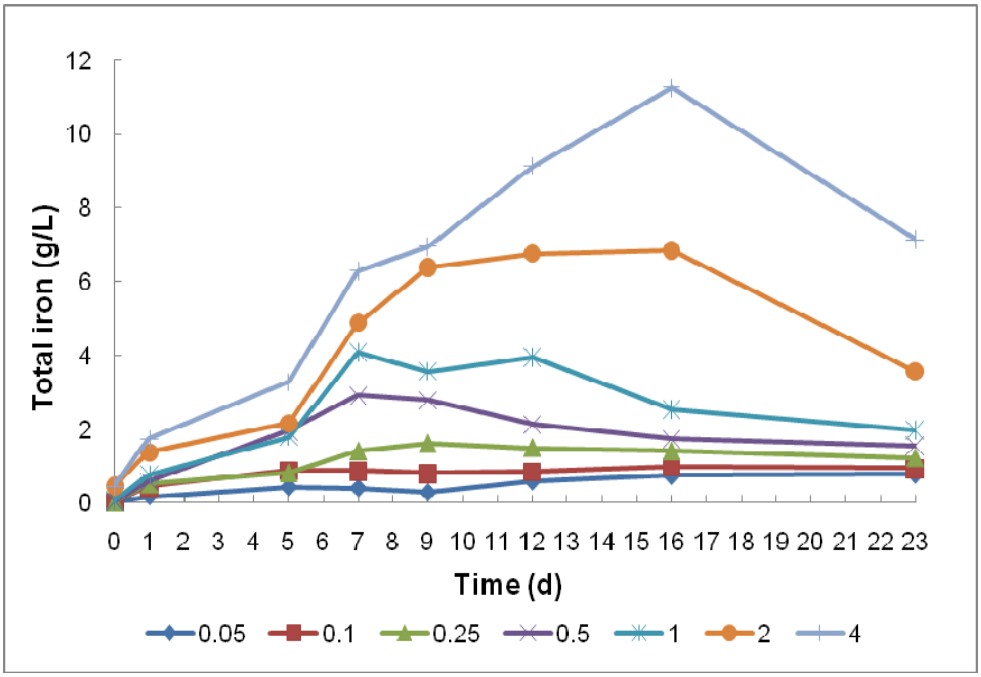
The quantity of total iron increased with increase in pyrite pulp density from 0.05% to 4% (Graph 6.15). In flasks having 0.05% and 0.1% P.D., maximum ferrous iron was leached in 5 days itself, after which total iron content of flask remained constant. Eighty six (86%) and 75% oxidation was achieved in 0.05% and 0.1% P.D. respectively by HGM 38, which decreased to 65% oxidation in 0.25% and 0.5% P.D. in 7 days. At 1, 2 and 4% pulp density of pyrite 48, 42 and 34.8% oxidation was observed in 16 days (Table 6.3). For HGM 38, maximum iron solubilization was achieved with 4% P.D, which was 11.26 g/l against 32.3 g/l present in the ore, which accounts for 34.8% leaching.

As can be seen from graph 6.16, rate of iron oxidation at lower concentrations of pyrite i.e. 0.05%, 0.1% and 0.25% increased sharply

Graph 6.14: Ferrous solubilization from pyrite by isolate HGM 38

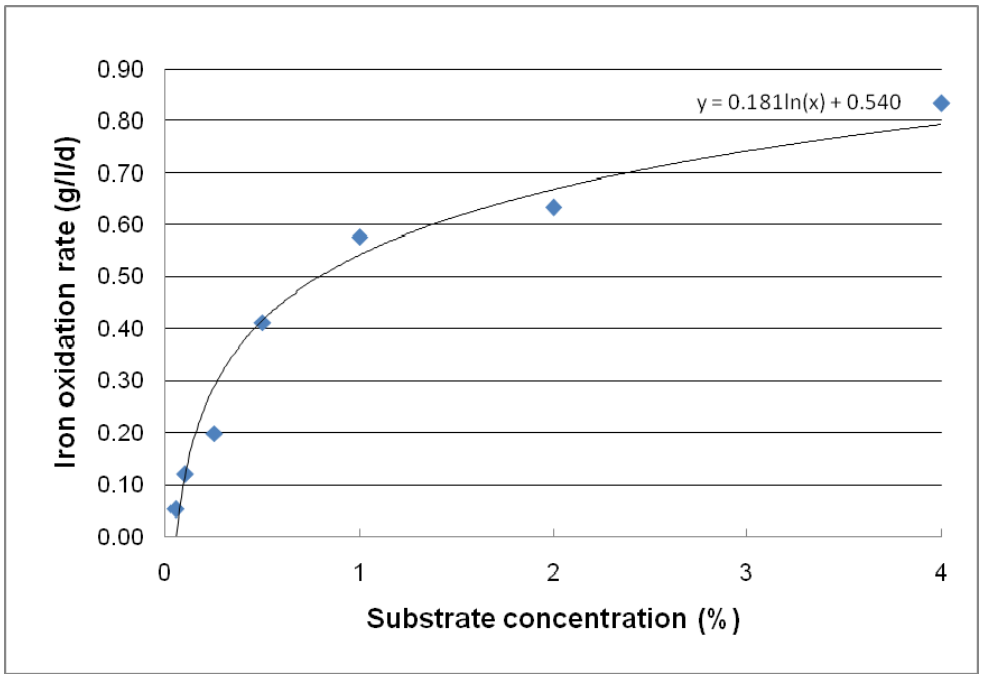


Graph 6.15: Dissolved total iron from pyrite by isolate HGM 38

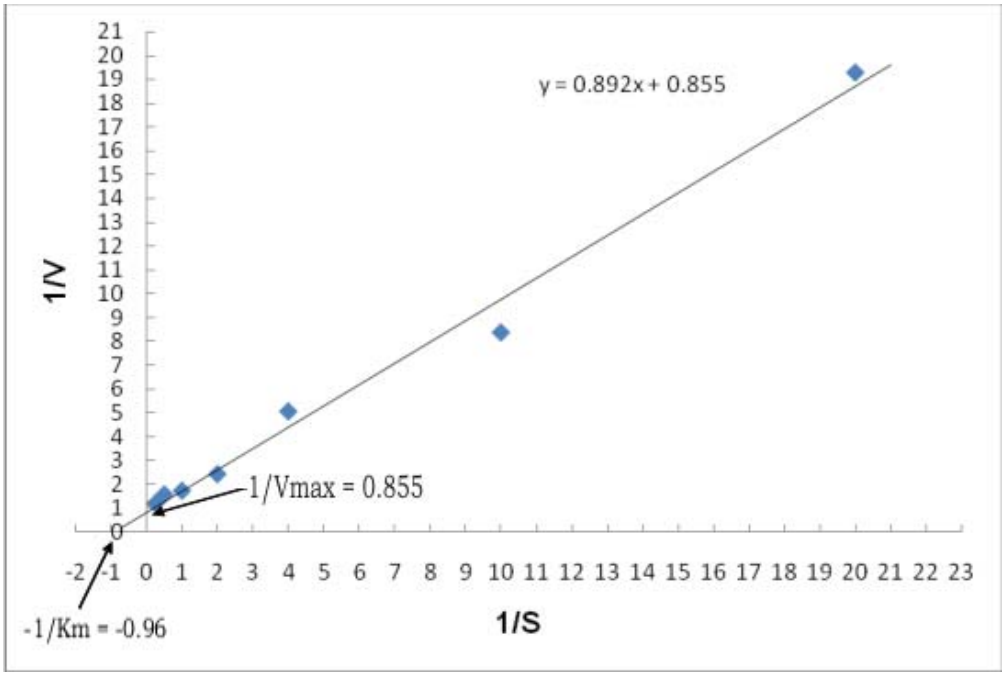


as compared to iron oxidation rate at higher pulp densities i.e. 2% and 4%. At 2% and 4% pulp densities the pyrite oxidation rate was 0.633 g/l/d and 0.833 g/l/d respectively. Reciprocal plot of pyrite oxidation rate (1/V) vs reciprocal of pulp density (1/S %) (graph 6.17) gave 1.04 K_m value and V_{max} was 1170 $mg.l^{-1}.d^{-1}$ or 48.75 $mg.l^{-1}.h^{-1}$.

Graph 6.16 : Rate of pyrite biooxidation by isolate HGM 38



Graph 6.17 : Reciprocal of Pyrite oxidation rate (1/V) vs reciprocal of pulp density (1/S%) – HGM 38



1/ V_{max} = 0.855	;	V_{max} = 1170 mg/l/d	
-1/Km = -0.96	;	Km = 1.04	

6.3.2.2 COMPARISON OF LEACHING BY ALL ISOLATES

Rate constants obtained during pyrite oxidation by all 4 isolates is given in table 6.2. Isolate SRD 5 has shown the least K_m value of 0.26 and highest maximum velocity (V_{max}) of 1320 mg/l/d among the four isolates tested. This makes SRD 5 the best isolate among four isolates, with respect to the rate of pyrite oxidation. However, when the extent of oxidation is concerned, as shown in graph 6.18 and table 6.3, HGM 30 is the isolate showing maximum oxidation of pyrite.

Table 6.3 shows the comparison of % oxidation obtained at each pulp density of pyrite at the end of experiment. In HGM 30, >99.5% oxidation was achieved in pulp densities from 0.05% to 0.5%, which is the highest pyrite oxidation extent among all the isolates. Moreover in 1% and 4% pyrite also, HGM 30 completed 90.78% and 56.68% oxidation in 30 days, while isolate HGM 26 showed highest oxidation in 2% pyrite containing flask, which was 91%. This is due to the fact that in isolate SRD 5, oxidation took place at the highest rate and it completed in 16 days, while in isolates HGM 26 and HGM 30, pyrite oxidation continued at a slower rate till 30 days. As a result in both these isolates higher amount of pyrite was oxidized. Least oxidation – 86 to 35% was observed with HGM 38 at all the pulp densities.

At lower concentrations of pyrite i.e. from 0.05% to 0.5% pyrite, SRD 5 showed good oxidation. However from 1% to 4% pulp density, SRD 5 showed less percent oxidation than HGM 26 and HGM 30. Higher oxidation might be achieved if isolate SRD 5 is adapted to ≥2% pulp density.

Table 6.2 : Rate constants of pyrite oxidation by 4 isolates

	K_m	V_{max} (mg Fe/L/d)
SRD 5	0.26	1320
HGM 38	1.04	1170
HGM 30	0.35	870
HGM 26	0.33	350

Graph 6.18 : Comparison of pyrite oxidation by various isolates

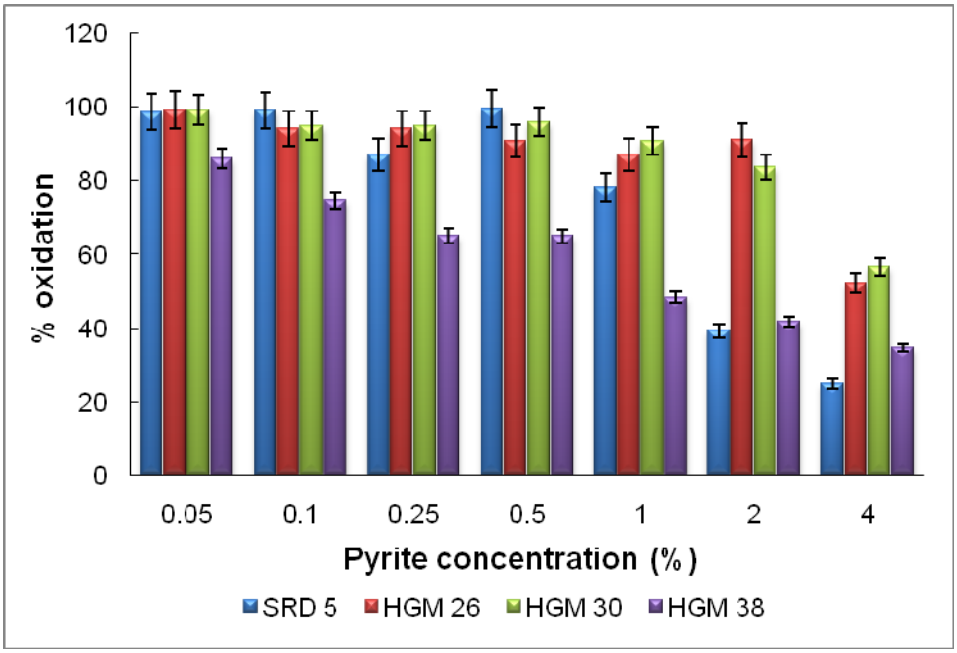


Table 6.3 : Percent oxidation achieved by 4 isolates

Pulp density	SRD 5	HGM 26	HGM 30	HGM 38
0.05	98.77	99.32	>99.5	86.1
0.1	99.15	94.36	>99.5	74.6
0.25	87	94.1	>99.5	65.07
0.5	99.7	90.81	>99.5	65
1	78.15	87.15	90.78	48.3
2	39.27	91	83.61	41.78
4	24.92	52.19	56.68	34.8

Pyrite oxidation rates shown by the four isolates is given in table 4.4. Isolates SRD 5 has given the highest oxidation rates at all pulp densities of pyrite except 2% PD. This makes isolate SRD 5 best pyrite oxidizer.

Table 6.4 : Pyrite oxidation rate achieved by 4 isolates

Pyrite concentration (%)	Pyrite oxidation rate ($g.l^{-1}.d^{-1}$)			
	SRD 5	**HGM 26**	**HGM 30**	**HGM 38**
0.05	0.228	0.062	0.105	0.052
0.1	0.292	0.042	0.264	0.119
0.25	0.518	0.121	0.280	0.198
0.5	1.091	0.301	0.295	0.411
1	1.204	0.546	0.779	0.577
2	1.404	0.726	1.656	0.633
4	1.511	0.821	1.050	0.833

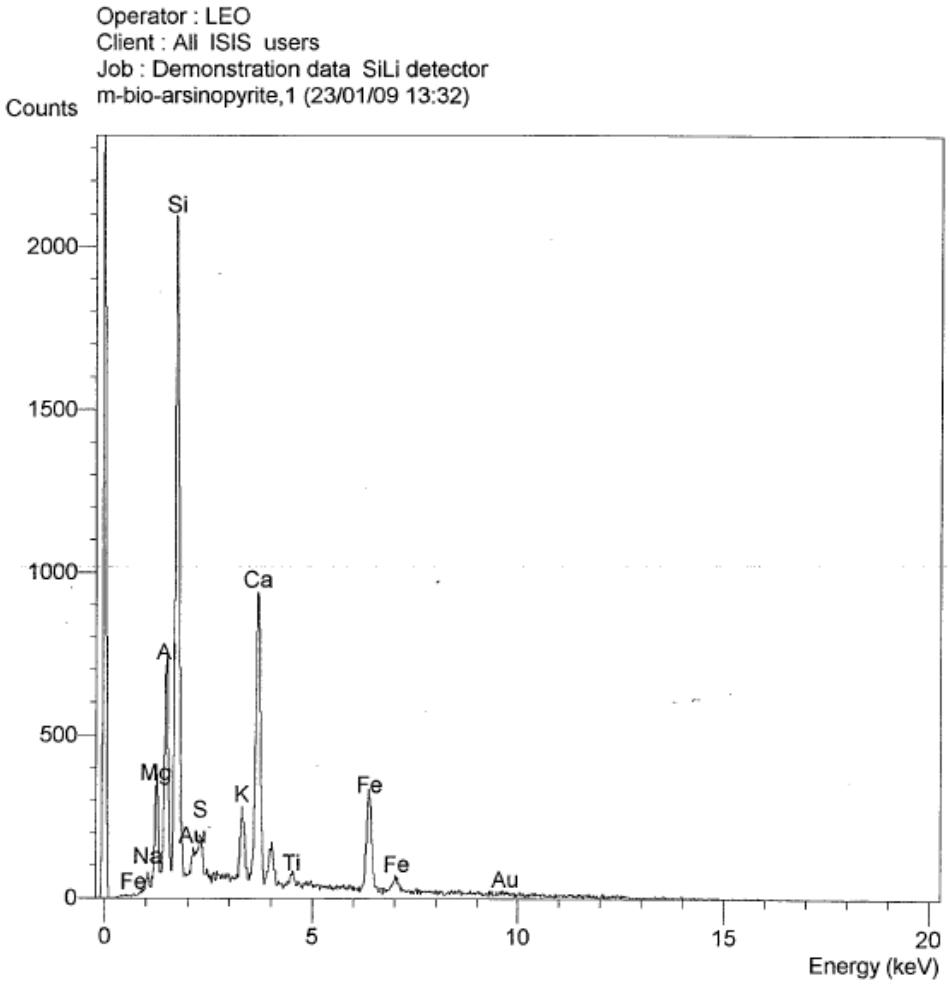
6.3.3 ARSENOPYRITE KINETIC STUDY

The amount of different components present in arsenopyrite ore was estimated by EDAX analysis and it is given in figure 6.2. This shows that silicon, calcium and aluminum are the main components of ore at 38.6%, 17.05% and 14.11% concentration. Iron constituted only 10.06% of the ore content.

As observed in graph 6.19, at all concentrations of arsenopyrite ore, dissolved ferrous iron concentration showed steep rise in 48 h. Between 48 h and 72 h, ferrous iron concentration in all pulp densities decreased. This may be due to increase in oxidation of soluble ferrous iron to ferric iron. After 72 h, the ferrous iron concentration again increased and in case of 4% and 8% PD, it continued upto 9 days. However the soluble ferrous did not reach upto even 1 g/l concentration during the experiment.

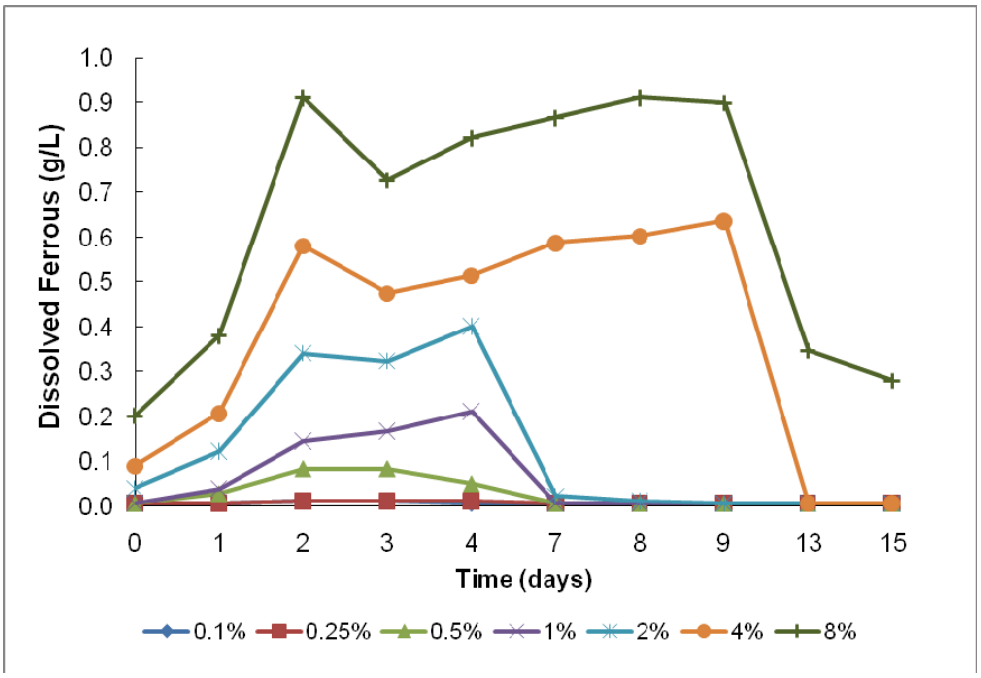
Total iron content in all the PD was equal to soluble ferrous content at the time of inoculation. However from 24^{th} h itself the total iron content increased 8 to 10 fold as compared to ferrous content. This shows that organisms were readily adapted to arsenopyrite at first exposure and no lag phase was observed (graph 6.20).

Figure 6.2 : Composition of refractory gold ore by EDAX analysis

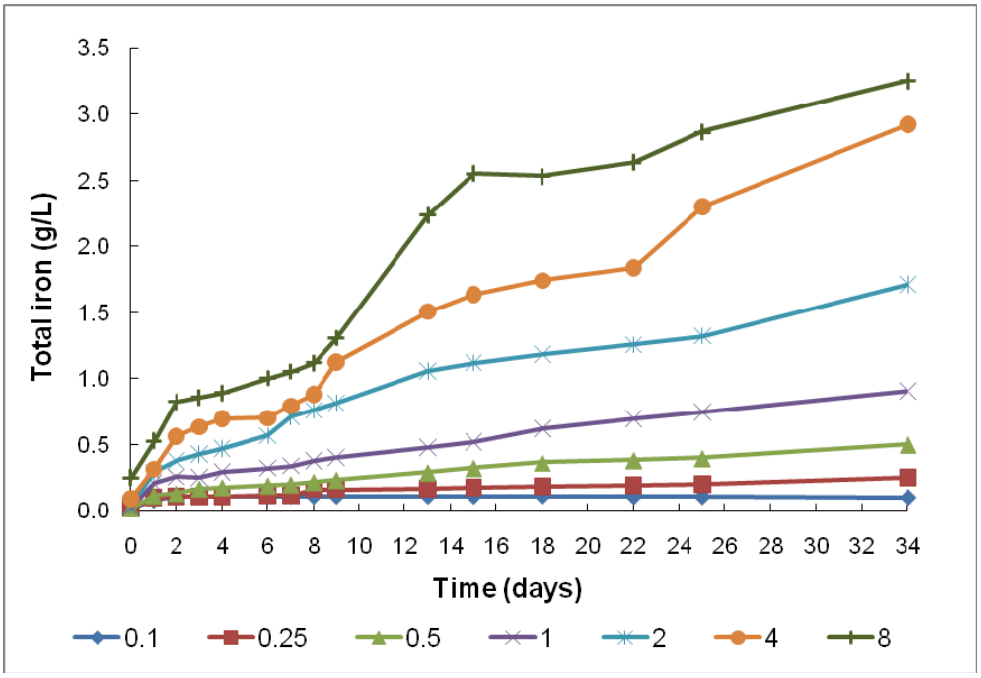


Element	Content (%)
Silicon (Si)	38.60
Calcium (Ca)	17.05
Aluminium	14.11
Iron (Fe)	10.06
Magnesium (Mg)	9.30
Potassium (K)	3.93
Sodium (Na)	2.80
Sulphur (S)	2.44
Titanium (Ti)	0.86
Gold (Au)	0.86
Total	**100.00**

Graph 6.19: Ferrous iron solubilized from aresenopyrite by SRD5

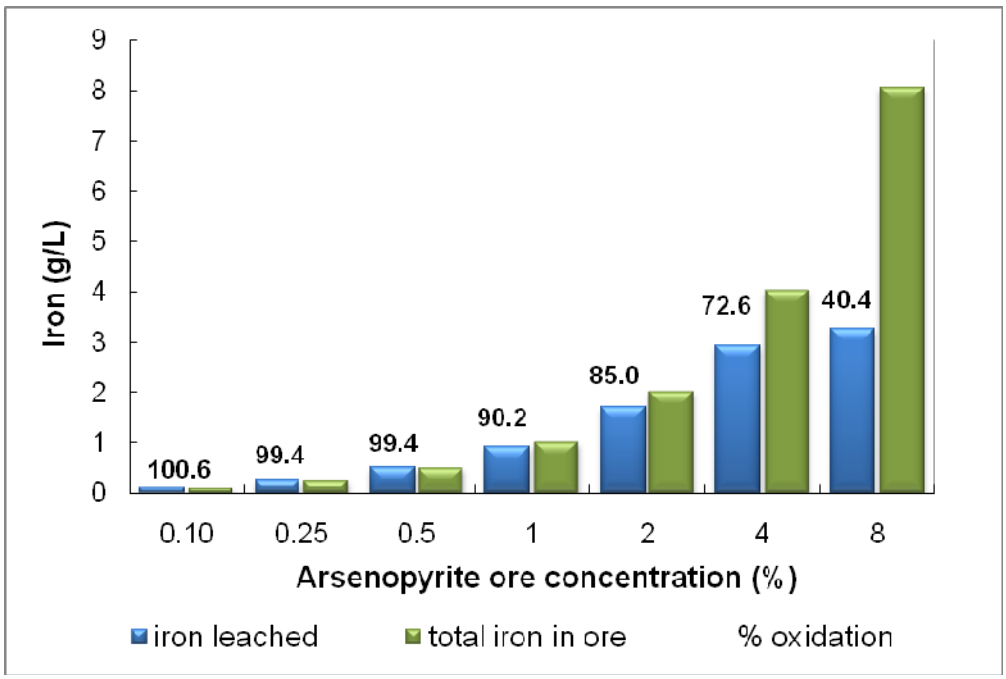


Graph 6.20 : Total iron solubilized from aresenopyrite by SRD 5



During initial 48 h, the total iron content showed rapid increase. After 48 h the rate of oxidation slowed down but consistent increase in total iron content was observed. This may be because of the fact that sufficient amount of arsenic was leached from arsenopyrite, which inhibited the leaching organism. Hence the slow increase in ferrous solubilization and total iron content was seen. After 8-9 d, possibly after the adaptation of organism to arsenic environment, the oxidation rate increased and continued upto the end of experiment.

Graph 6.21: Percentage of iron leached from arsenopyrite as compared to total iron present



At lower concentrations of arsenopyrite i.e. 0.05% and 0.1% PD more than 99% oxidation could be achieved, while 0.25% PD showed 89% extraction (graph 6.21). 0.5, 1 and 2% ore gave considerable fair oxidation in the first exposure itself, which was 59%, 41% and 35% respectively.

Nyashanu *et al.* (1999) reported that the oxidation of As^{+3} was found to be catalyzed by pyrite surfaces and bacteria in presence of Fe^{+3}. However, Fe^{+3} alone was not able to oxidize As^{+3}. Adding pyrite and Fe^{+3} to arsenopyrite concentrate promotes the formation of As^{+5} leaving less than 1% of the extracted arsenic as As^{+3}.

7.1 INTRODUCTION

Presently more genomic sequences of different bioleaching organisms are deciphered, it is being increasingly possible to differentiate between members of a same genera and even species. The bioleaching of metals from sulfidic ore is carried out by a consortium of acidophilic, autotrophic microorganisms, among which strains of *Acidithiobacillus ferrooxidans, Acidithiobacillus thiooxidans and Leptospirillum ferrooxidans* play a predominant role (Sand *et al.*, 1992; Velenzuela *et al.* 2006). Apart from these dominant organisms, several other microorganisms are also present in mining environment which could not be identified because of their requirement of specific enrichment method or culturing conditions (Jerez 2008).

One of the most studied organisms that thrives in acidic mining sites is *Acidithiobacillus ferrooxidans* formerly *Thiobacillus ferrooxidans* (Kelly and Wood, 2000). This is a gram negative chemolitho-autotrophic bacterium that obtains energy from the oxidation of ferrous iron, elemental sulfur, reduced sulfur compounds and sulfide minerals. It has been considered as a model biomining organism (Ageeva *et al.* 2003). Since their natural habitat are ecological extremely diverse, different *Acidithiobacillus* strains of the same species developing in various ecological niches are characterized by differences in growth rate, tolerance to heavy metal ions and activities of ferrous iron and/or sulfide mineral oxidation (Ageeva *et al.*, 2001; Kondrat'eva *et al.*, 2002; Ni *et al.* 2008). They also vary in their genetically determined ability to adapt to other oxidation substrates. Various other characteristics, such as optimum growth temperature, response to organic carbon sources and cellular morphologies facilitate, in some cases, identification of isolate to a genus or species level (Johnson *et al.* 2005).

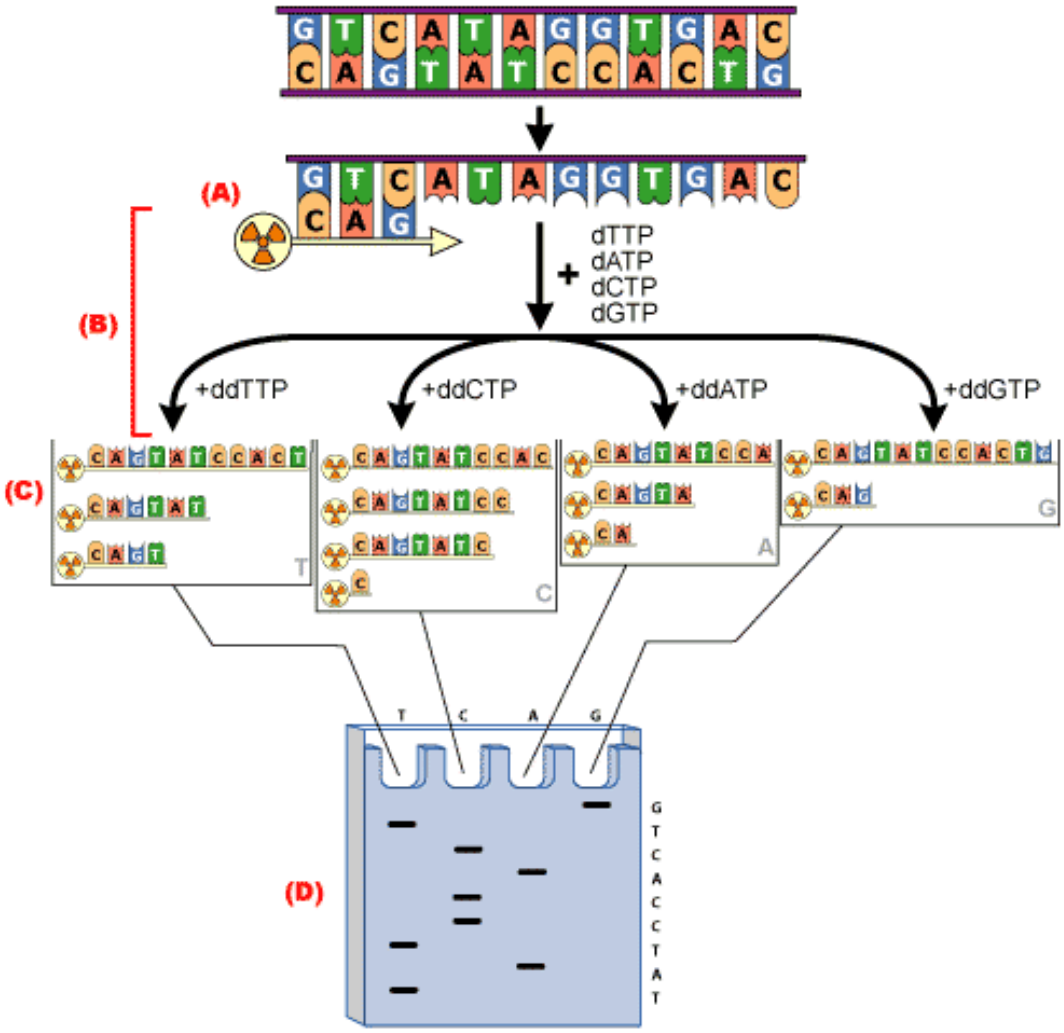
The species of *A. ferrooxidans* is characterized by a wide range of inter-strain genotypic variability with respect to the genome size, DNA G+C content, level of DNA-DNA homology of total genomes, chromosomal DNA

structure and number and size of plasmids as revealed by genetic analysis methods (Kondrat'eva *et al.*, 2002). The most common techniques employed to explore bacterial diversity use culture independent methods like 16S rRNA and –rDNA profiles, fluorescent in situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), Real time PCR, ARDRA, RAPD, AFLP and others (Vos *et al.*, 1995; Janssen *et al.*, 1996; Kodama and Watanabe, 2003; Okibe *et al.*, 2003; Bruneel *et al.*, 2006; Zhang *et al.*, 2007; Rusch and Amend, 2007). Figure 7.1 shows the principle by which DNA or RNA strands are sequenced.

Due to the development of new techniques for the study of molecular microbial ecology, many new *Acidithiobacillus* -related and *Leptospirillum*–related bacteria have been characterized (Yates and Holmes, 1987; Coram and Rawlings, 2002; Karavaiko *et al.*, 2003; Demergasso *et al.*, 2005; Bruscella *et al.*, 2005; Ni *et al.*, 2008 and Yin *et al.*, 2008).

Pizarro *et al.* (1996) analyzed the spacer region between 16 and 23S gene in bacterial rRNA genetic loci to identify organism present in a mixture of copper bioleachate. Selenska *et al.* (1998) studied RFLP analysis of PCR amplified 16S rDNA, 23S rDNA, and intergenic spacer rDNA between the 16S and the 23S rRNA-genes (amplified ribosomal DNA restriction enzyme analysis – ARDREA), as well as genomic fingerprinting using random primers (RAPD) and repetitive primers (Rep-APD) (Ni *et al.*, 2008). The taxonomic and discriminatory power of these three analytical approaches was compared. It was demonstrated that the taxonomic results obtained by RAPD and Rep-APD methods were in agreement with the RFLP analysis. But in contrast to ARDREA, RAPD method derives information from the whole bacterial genome, where regions with higher variability are present than those of the rRNA-genes (Selenska-Pobell *et al.* 1998). By them, a discrimination of closely related *Acidithiobacillus* strains was possible. In addition, RAPD and Rep-APD are easier to perform and faster.

Fig 7.1 : Principle of DNA sequencing



Each *A. ferrooxidans* strain possesses a unique endonuclease restriction pattern of the chromosomal DNA, and this provides a means for its identification among other strains and its monitoring in nature, in biotechnological processes and under changed cultivation conditions (Kondrat'eva *et al.*, 2002).

Random Amplified Polymorphic DNA (RAPD) analysis described by Williams *et al.* (1990) is a commonly used molecular marker in genetic diversity studies. Other related techniques include Arbitrary Primed PCR (AP-PCR) and DNA Amplification Fingerprinting (DAF). These methods differ from RAPDs in primer length, the stringency conditions and the method of separation and detection of the fragments.

7.1.1 RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD)

Analysis of randomly amplified polymorphic DNA (RAPD) is widely used to investigate variability among microorganisms. This technique is fast, cheap and easy to perform, and requires only small amounts of DNA that is available even from dried materials and does not require any previous sequence information. In RAPD, agarose gel electrophoresis and ethidium bromide (EtBr) staining are used for DNA detection. RAPD involves the use of arbitrary GC-rich decamers as single primers instead of reverse and forward primers to amplify unknown sites in the target genome. During the last several years, RAPD has been developed and applied for the characterization of bacteria and their classifications, groupings, identification and placement on the level of strains. Moreover, RAPD analysis is based upon PCR, which is a very sensitive technique and should give reproducible results (Akbar *et al.* 2005).

7.1.2 THEORY UNDERLYING RAPD MARKERS

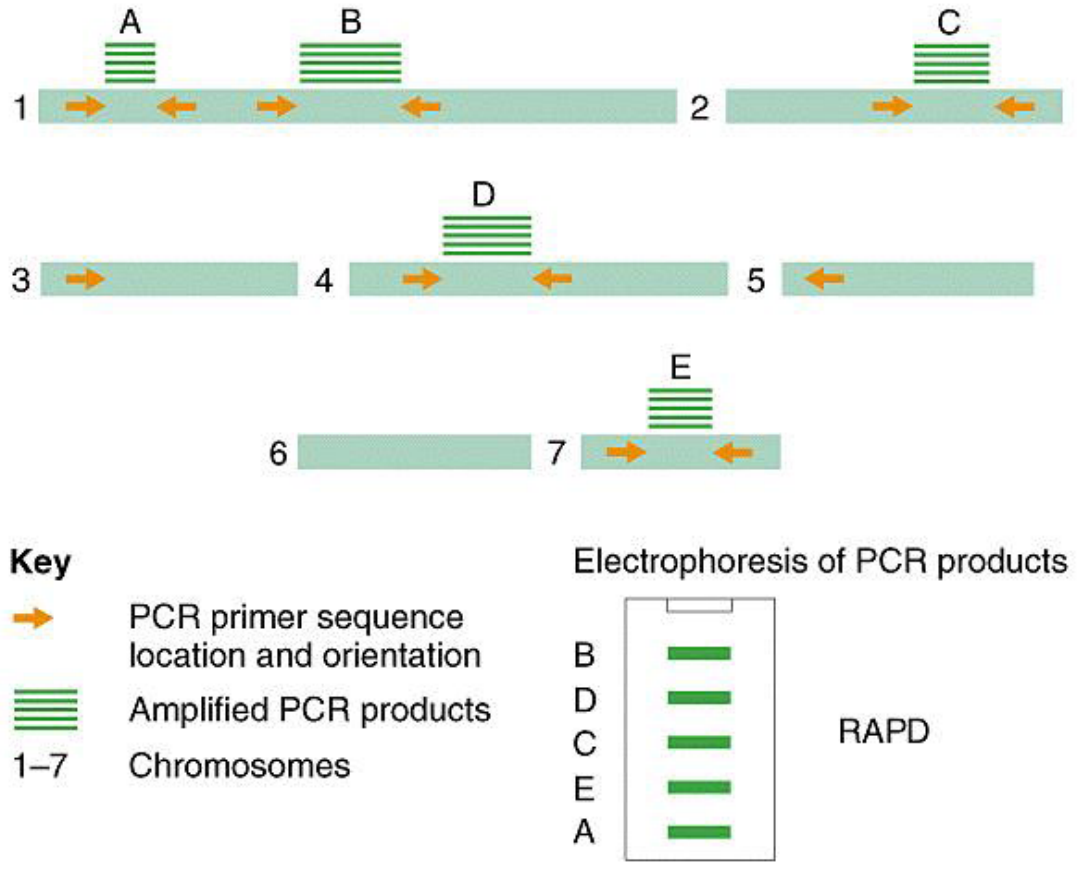
The principle involved in generating RAPDs is that, a single, short oligonucleotide primer, which binds to many different loci, is used to amplify random sequences from a complex DNA template. This means that the amplified fragments generated by PCR depend on the size of both

the primer and the target genome. The assumption is made that a given DNA sequence (complementary to that of the primer) will occur in the genome, on opposite DNA strands, in opposite orientation within a distance that is readily amplifiable by PCR. These amplified products (of up to 3.0 kb) are usually separated on agarose gels and visualized by ethidium bromide staining (Selenska-Pobell *et al.* 1998; Bardacki, 2001). As shown in figure 7.2, The use of a single 10-mer oligonucleotide promotes the generation of several discrete DNA products and these are considered to originate from different genetic loci. Polymorphisms result from mutations or rearrangements either at or between the primer binding sites and are detected as the presence or absence of a particular RAPD band (Selenska-Pobell *et al.* 1998). This means that RAPDs are dominant markers and therefore cannot be used to identify heterozygotes.

RAPDs have been used widely because of the following advantages:

1. RAPDs require no DNA probes and sequence information is not required for the design of specific primers.
2. The procedure involves no blotting or hybridization steps. The technique is therefore quick, simple and efficient.
3. RAPDs require small amounts of DNA (about 10 ng per reaction) and the procedure can be automated. RAPDs have also been reported to detect higher levels of polymorphism compared, for example, with RFLPs in cases where the two have been applied to the same material.

Fig 7.2: General model of RAPD analysis



7.2 MATERIALS AND METHODS

7.2.1 EXTRACTION AND PURIFICATION OF GENOMIC DNA

The genomic DNA of each of the 8 cultures was isolated and purified by Axygen miniprep DNA purification kit. Purified genomic DNA extracts from the isolates were used as template DNA. The purity of genomic DNA was measured on 1.2% agarose gel in TBE buffer. The purified DNA was quantified by optical density in Nanodrop spectrophotometer model ND-1000 and analyzed by software ND-1000-V3.3.0.

7.2.2 RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) PROFILING

A set of 20 random decamer primers were used in RAPD analysis. Each primer was employed to study polymorphism between 8 isolates by RAPD-PCR method in which purified genomic DNA from all the isolates served as template DNA. RAPD mastermix was prepared afresh using 5X assay buffer without $MgCl_2$ (5 µl, Promega USA), 25 mM $MgCl_2$ (1.5 µl), 2 mM dNTPs (2 µl, Promega USA), 1 µl primer (100 pmol/ µl), 1 µl Taq DNA polymerase (5 u/µl, Promega USA) and 11.5 µl PCR water for all the isolates to avoid handling errors. Twenty decamer DNA primers were procured from Operon Biotechnologies, GMBH, Cologne, Germany and were made up in 0.5X PCR buffer with KCl (Fermentas, USA). The mastermix was distributed in 8 microfuge tubes of 0.5 ml capacity for each of the 20 primer. In each reaction, only one primer was used which alone acted as both forward and reverse primer and amplified those segments of DNA which fall between the primers.

The PCR mastermix along with the primer made volume upto 22 µl, which when added with 3 µl of template DNA from any 1 of the 8 isolates completed the total reaction volume in each tube to 25 µl. PCR amplification was done in Mastercycler (Eppendorf AG, Germany) by following parameters.

I stage: one cycle of denaturation at 94°C for 4 min.

II stage : 35 cycles consisting of (i) denaturation at 94°C for 1 min. (ii) annealing at 37°C for 3 min. and (iii) extension at 72°C for 1 min. and

III stage: one cycle of extension at 72°C for 10 min. and held at 4°C till further process.

Once the required cycles of amplification were compete, the PCR product were loaded onto 1.2% agarose gel in Tris-borate-EDTA buffer and run at 150 V per gel for 4 h. A DNA ladder of 100-1000 bp (G-biosciences, USA) was also run along with amplicons of each primer on the same gel. The gel with amplification profiles was visualized under UV transilluminator, analyzed by gel documenting system (Ultra Violet Products, Korea) using gel analysis software TL120 (Total lab Ltd., Nonlinear Dynamics, UK).

Each primer reaction was run in duplicate to ensure reproducibility of the amplification reaction. The bands of amplified DNA fragments were compared and marked for presence (1) and absence (0) of each reproducible DNA band. Molecular weight of each band was identified by software on the basis of DNA marker. Individual band from each isolate if fell within 0.1 point Rf value, it was considered as common band for all isolates. The list of all the primers and its sequences is given in table 7.1.

Statistical analysis

Nei and Li's coefficient was used to calculate the relatedness of the studied species among each other. Nei and Li's co-efficient was calculated by the following statistical equation

$$F = 2Nxy / (Nx + Ny)$$

where F is the similarity co-efficient,

Nx and *Ny* are the number of fragments from population *x* and *y*, resp. *Nxy* is the number of fragments shared by the two populations.

Table 7.1: Primers used in the RAPD analysis

Sr. No.	Primer	Sequence
1	OPA 2	TGC CGA GCT G
2	OPA 3	AGT CAG CCA C
3	OPA 4	AAT CGG GCT G
4	OPA 5	AGG GGT CTT G
5	OPA 6	GGT CCC TGA C
6	OPA 7	GAA ACG GGT G
7	OPA 8	GTG ACG TAG G
8	OPA 10	GTG ATC GCA G
9	OPA 11	CAA TCG CCG T
10	OPA 12	TCG GCG ATA G
11	OPA 13	CAG CAC CCA C
12	OPA 14	TCT GTG CTG G
13	OPA 15	TTC CGA ACC C
14	OPA 16	AGC CAG CGA A
15	OPA 17	GAC CGC TTG T
16	OPA 18	AGG TGA CCG T
17	OPA 19	CAA ACG TCG G
18	OPA 20	GTT GCG ATC C

The data generated by RAPD was analyzed with software Free Tree 0.9.1.50. A similarity matrix was generated using Nei and Li's coefficient of similarity and a dendrogram was generated using Unweighted Pair Group Method of Arithmetic Means (UPGMA). Polymorphism observed within 8 isolates for each marker was calculated as per Megha *et al.* (2007).

7.2.3 16S rRNA GENE SEQUENCING

Full length 16SrRNA gene fragment was amplified by PCR from above isolated genomic DNA. Sequencing of purified PCR amplicon was carried out and sequence data was processed. Screening of single sequences for chimeras and other anomalies was done using Pintail online software (http://www.bioinformatics-toolkit.org/Web-Pintail/). Pintail is a tool for identifying anomalies, including chimeras, within 16S rRNA sequences. The program works by comparing evolutionary distances between a query and subject sequence over the length of the 16S rRNA gene (small subunit rRNA), by employing a sampling window of specified size, progressing a fixed number of bases at a time along the length of the gene. The final sequences were initially analyzed using the BLAST search facility (www.ncb.nlm.nih.gov/blast/blast.cgi) and identity of the organisms were confirmed with the 16S rDNA of standard Type strain available at EzTaxon server 2.1(147.47.212.35/8080). Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* software version 4 (Tamura *et al.* 2007). Phylogenic tree reconstruction analysis was done by UPGMA method with 1000 replication bootstrap value.

7.3 RESULTS AND DISCUSSION

7.3.1 EXTRACTION AND PURIFICATION OF GENOMIC DNA

Genomic DNAs of all the 8 isolates were isolated from the purified culture pellets, which were washed twice with 0.1 N HCl to remove the traces of ferric iron. The pellets were then washed with 7.0 pH milli-Q water 2-3 times, before subjecting to DNA isolation. The quality of isolated DNAs were analyzed on 1.2% agarose gel in TBE buffer, where DNA from each isolate showed a single band of high molecular weight DNA on 1.2% agarose gel. The quantity of each DNA purified from isolate along with absorbance ratio is given in table 7.2. The molecular weight of isolated DNAs ranged from 11.4 to 37.5 (ng/µl) and its purity value of 1.93 to 2.18 $A_{260/280}$. Since the 260/280 absorbance ratio of all the extracted DNA was above 1.9, it indicated higher purity of all the DNAs. While the

260/230 absorbance ratio of 4 isolates HGM 30, 2MC, E' and Lig-tf was found to be more than 1.3. Ideally less 260/230 absorbance is an indication of less contamination from RNA.

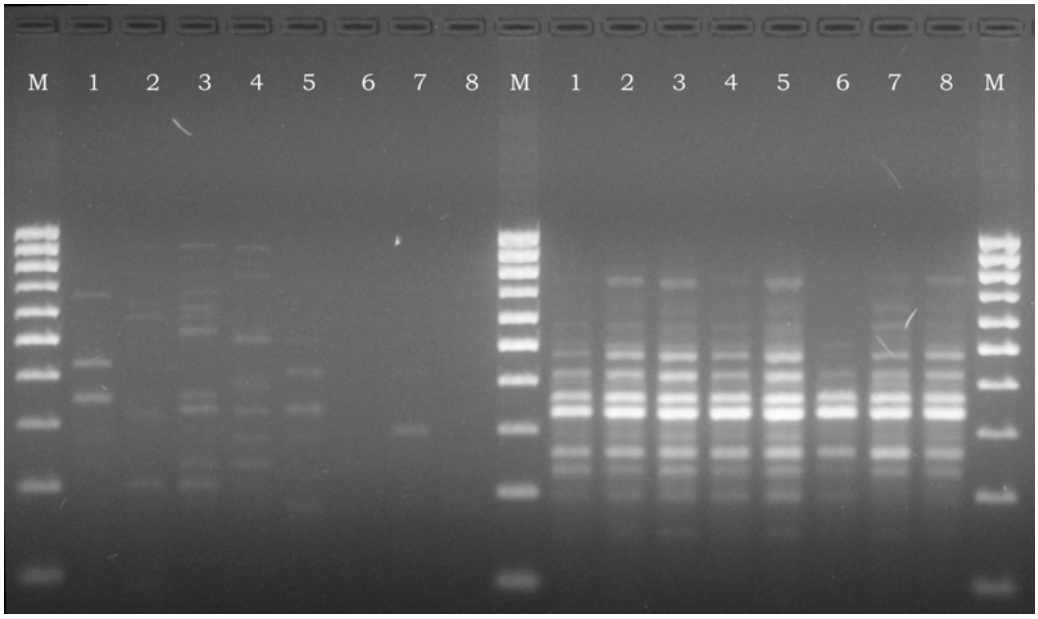
Table 7.2: Spectrophometric analysis of purified DNA

Sr. No.	Isolate	Quantity of DNA (ng/μl)	Absorbance ratio	
			260/280	260/230
1	SRD 5	23.5	2.18	0.41
2	HGM 17	37.5	1.96	0.62
3	HGM 26	20.3	1.92	0.65
4	HGM 30	27.3	1.97	1.98
5	HGM 38	24.5	2.14	0.28
6	E'	11.4	2.01	1.3
7	2MC	32.4	1.98	1.74
8	Lig Tf	28.2	1.93	1.5

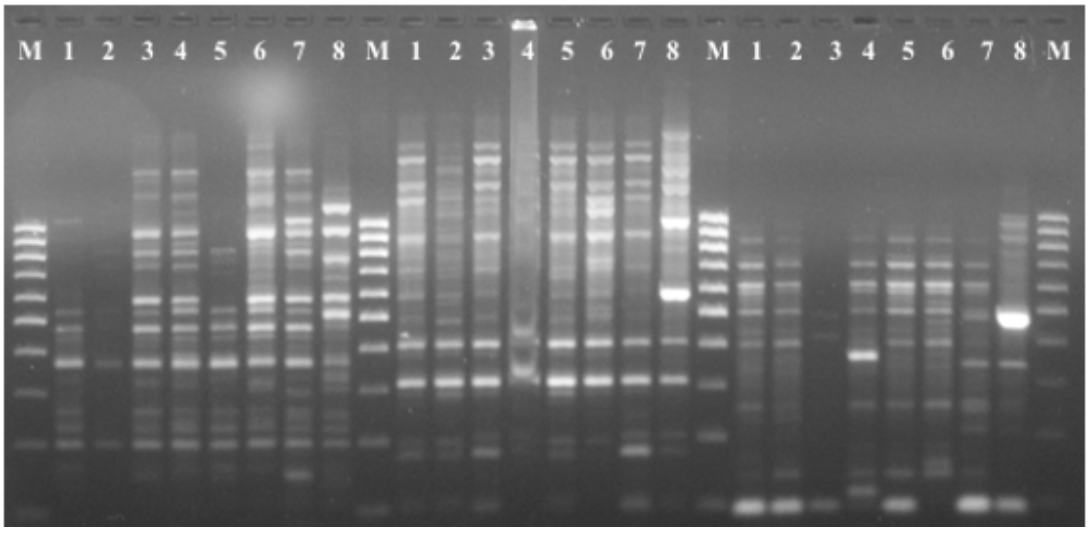
7.3.2 RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) PROFILING

Individual RAPD primer can hybridize to several hundred sites on test DNA but when they anneal at two sites on opposite strands of DNA within 2kb of each other amplification occurs and fragments are generated. These fragments are usually generated from different regions of the genome and hence multiple portions of test DNA can be analyzed very quickly (Edwards 1998). Genomic DNA of 8 isolates were amplified using 20 decamer primers OPA 1 to OPA 20. Eighteen primers gave reproducible and clear bands, while two primers OPA 1 and OPA 9 failed to give scorable bands. Amplification of DNA of all the 8 isolates with each primer gave heterogenous RAPD profiles, which gave a remarkable insight into the differences present in DNA sequences. The RAPD profiles of all the 8 isolates obtained with the 20 primers is shown in photograph 7.1 to 7.7.

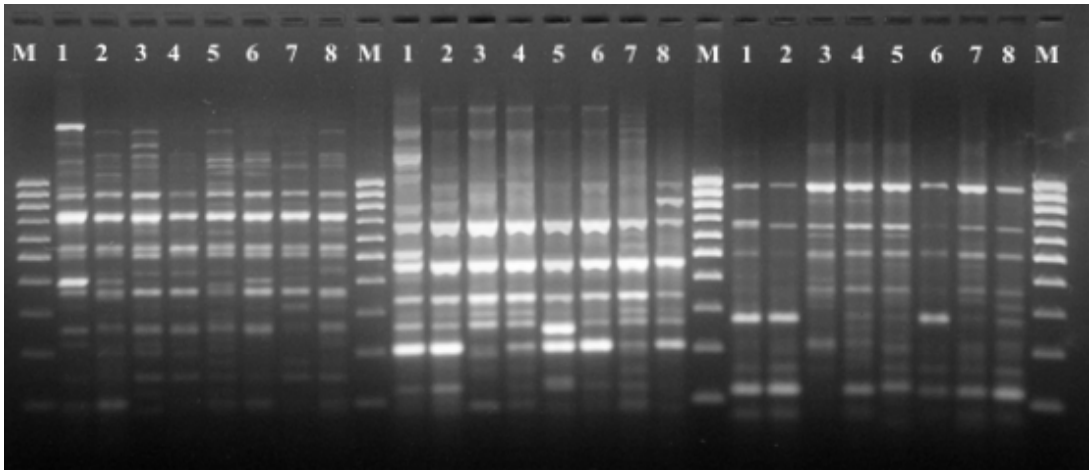
Photograph 7.1 : RAPD profiles of 8 *Acidithiobacillus ferrooxidans* isolates on 1.5% Agarose gel in TBE buffer, generated with primers OPA-01 and OPA-02. M : DNA marker (100bp – 1000 bp ladder), 1 : SRD 5, 2: Isolate HGM 17, 3: Isolate HGM 26, 4: Isolate HGM 30, 5 : Isolate HGM 38, 6 : Isolate E', 7 : Isolate 2MC, 8 : Isolate Lig Tf



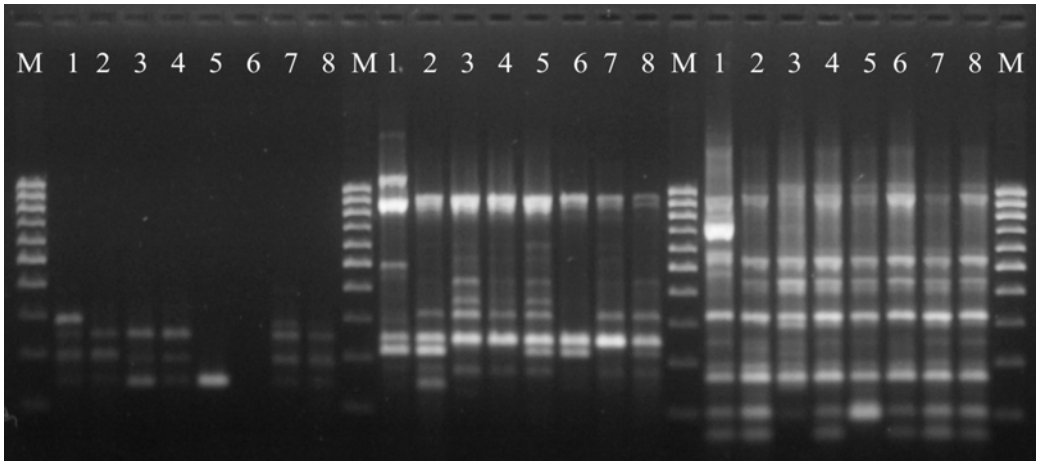
Photograph 7.2 : RAPD profiles of 8 *Acidithiobacillus ferrooxidans* isolates on 1.5% Agarose gel in TBE buffer, generated with primers OPA-03, OPA-04 and OPA-05.



Photograph 7.3 : RAPD profiles of 8 *Acidithiobacillus ferrooxidans* isolates on 1.5% Agarose gel in TBE buffer, generated with primers OPA-06, OPA-07 and OPA-08.

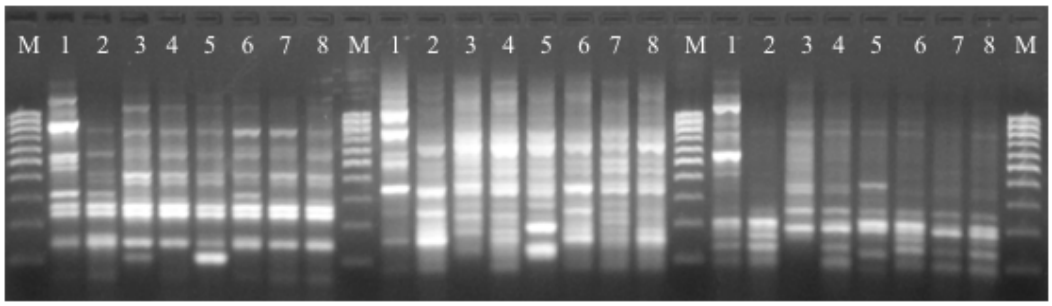


Photograph 7.4 : RAPD profiles of 8 *Acidithiobacillus ferrooxidans* isolates on 1.5% Agarose gel in TBE buffer, generated with primers OPA-09, OPA-10 and OPA-11.

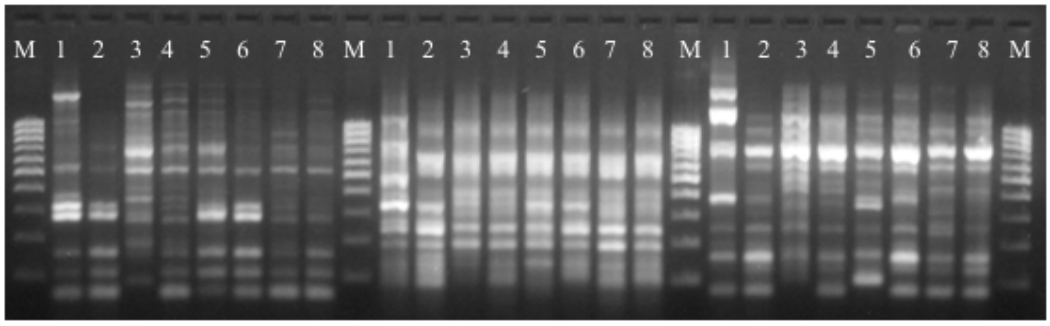


M – DNA marker (100bp – 1000 bp ladder)	
1 – SRD 5	5 – Isolate HGM 38
2 – Isolate HGM 17	6 – Isolate E'
3 – Isolate HGM 26	7 – Isolate 2MC
4 – Isolate HGM 30	8 – Isolate Lig Tf

Photograph 7.5 : RAPD profiles of 8 *Acidithiobacillus ferrooxidans* isolates on 1.5% Agarose gel in TBE buffer, generated with primers OPA-12, OPA-13 and OPA-14.

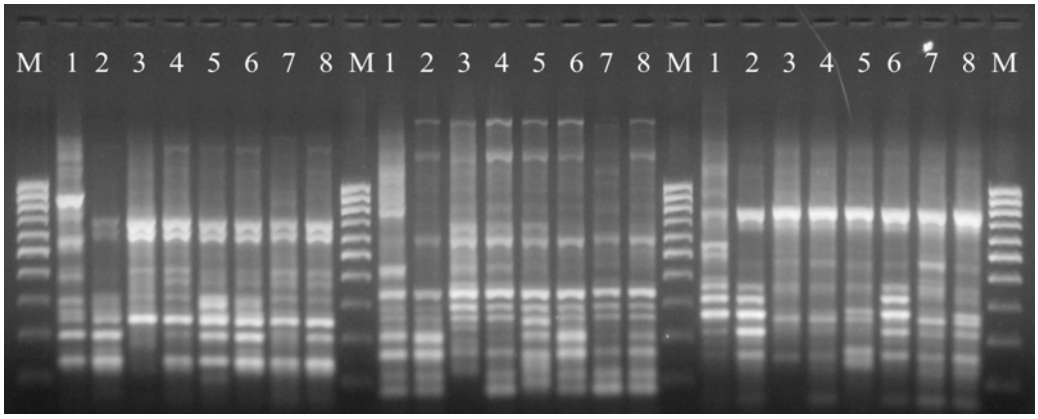


Photograph 7.6 : RAPD profiles of 8 *Acidithiobacillus ferrooxidans* isolates on 1.5% Agarose gel in TBE buffer, generated with primers OPA-15, OPA-16 and OPA-17.



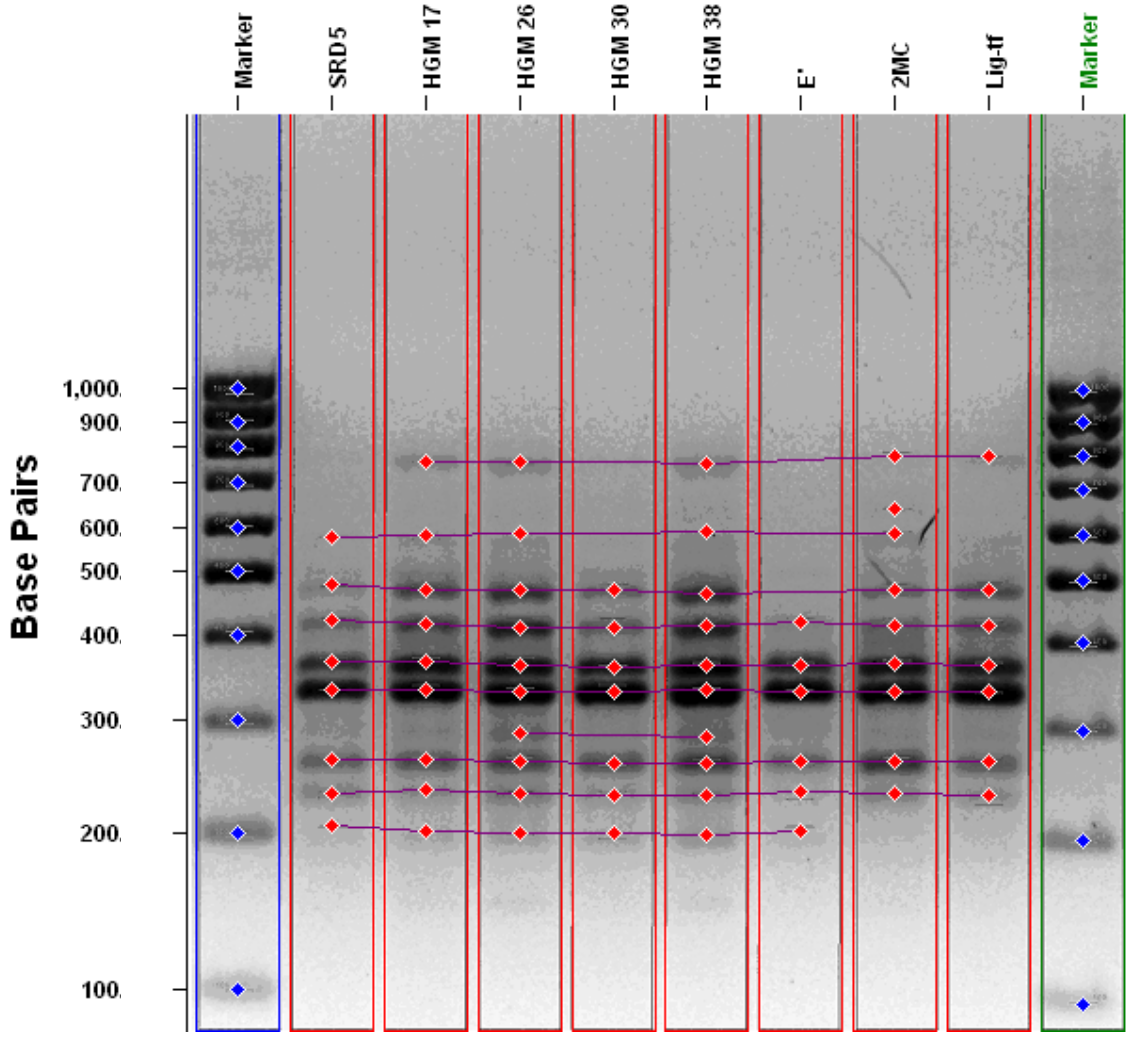
M – DNA marker (100bp – 1000 bp ladder)	
1 – SRD 5	5 – Isolate HGM 38
2 – Isolate HGM 17	6 – Isolate E’
3 – Isolate HGM 26	7 – Isolate 2MC
4 – Isolate HGM 30	8 – Isolate Lig Tf

Photograph 7.7 : RAPD profiles of 8 *Acidithiobacillus ferrooxidans* isolates on 1.5% Agarose gel in TBE buffer, generated with primers OPA-18, OPA-19 and OPA-20.



M – DNA marker (100bp – 1000 bp ladder)	
1 – SRD 5	5 – Isolate HGM 38
2 – Isolate HGM 17	6 – Isolate E'
3 – Isolate HGM 26	7 – Isolate 2MC
4 – Isolate HGM 30	8 – Isolate Lig Tf

Fig 7.3 : Analysis of RAPD bands obtained by OPA 2



As shown in figure 7.3, the fragment profile generated from DNA sample of all 8 isolates with primer OPA2 contained 6 to 11 bands of variable intensity which ranged in size from 214-765 bp. Out of total 11, 6 bands were found to be polymorphic giving 54% polymorphism. Out of total 8 isolates subjected to RAPD-PCR by primer OPA 2, 7 different phenotypes were observed. Therefore OPA 2 could differentiate 87% of all isolates. HGM 26 and HGM 38 gave 100% similar phenotype as seen in figure 7.4. Many strains of *Acidithiobacillus ferrooxidans* have been described in the literature, all sharing the more or less uniform phenotype of being acidophilic, obligately chemolithoautotrophic, Gram negative rods that use the oxidation of ferrous iron for energy generation but they exhibit

considerable genetic variation. By PCR based techniques, similarity coefficient between various isolates were obtained which ranged from almost 0% to over 98% indicating the genomic variability (Kelly and Wood, 2000).

Fig 7.4 : Dendrogram of isolates as obtained by OPA 2

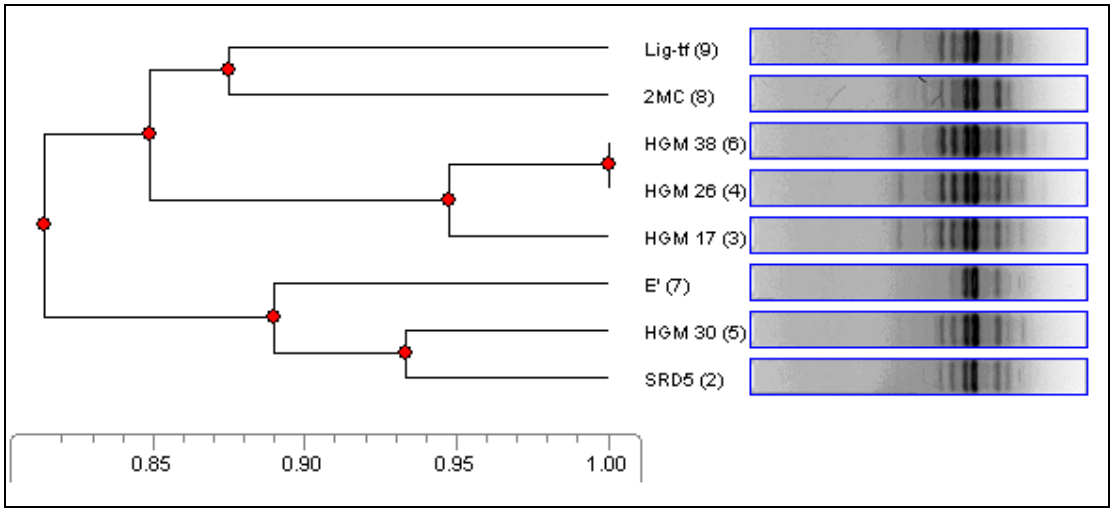
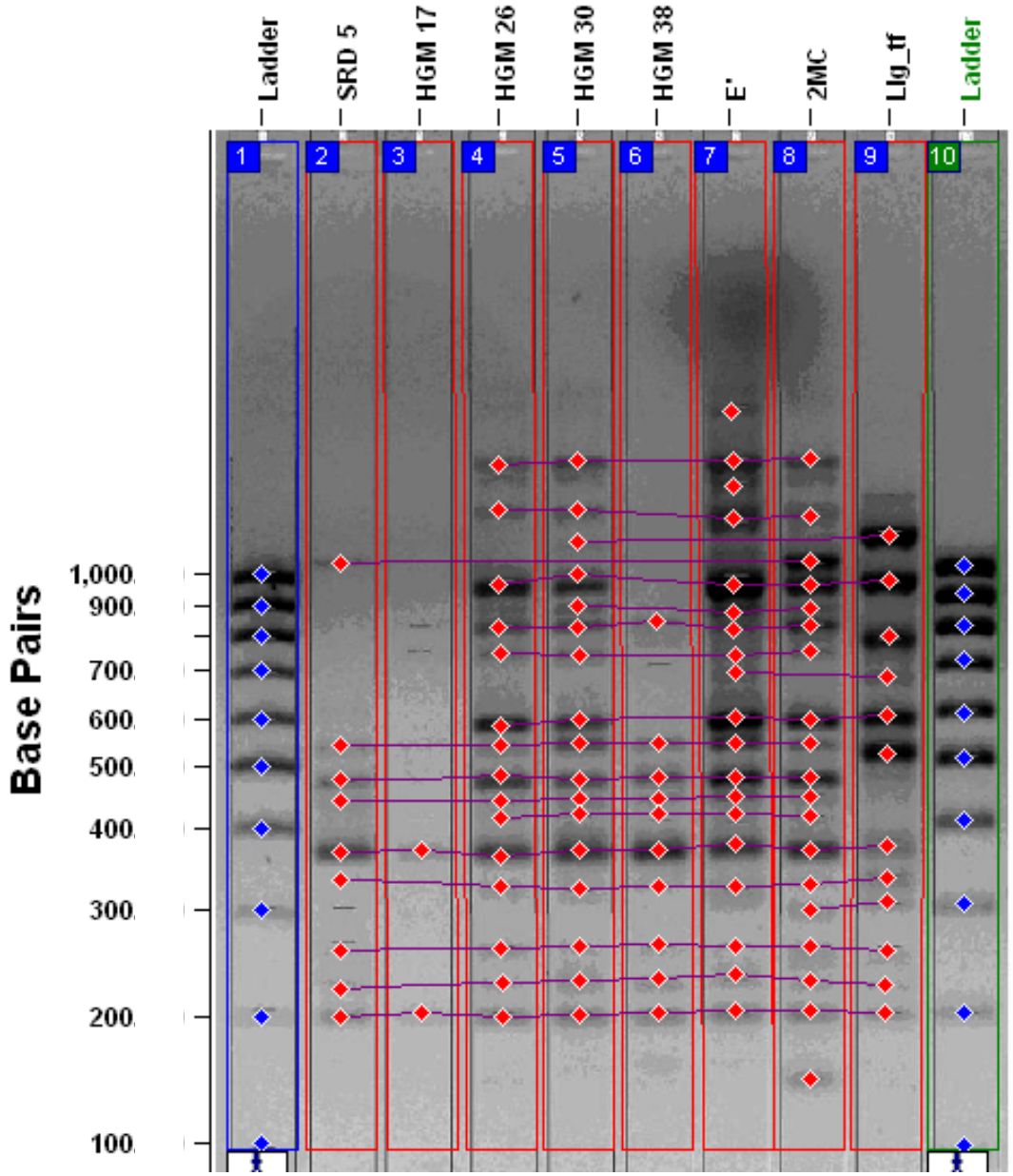


Table 7.3: Similarity matrix given by OPA 2

Lane	SRD 5	HGM 17	HGM 26	HGM 30	HGM 38	E'	2MC	Lig tf
SRD 5	1	0.94	0.89	0.93	0.89	0.86	0.82	0.8
HGM 17	0.94	1	0.95	0.88	0.95	0.8	0.89	0.88
HGM 26	0.89	0.95	1	0.82	1	0.75	0.84	0.82
HGM 30	0.93	0.88	0.82	1	0.82	0.92	0.75	0.86
HGM 38	0.89	0.95	1	0.82	1	0.75	0.84	0.82
E'	0.86	0.8	0.75	0.92	0.75	1	0.67	0.77
2MC	0.82	0.89	0.84	0.75	0.84	0.67	1	0.88
Lig tf	0.8	0.88	0.82	0.86	0.82	0.77	0.88	1

As shown in table 7.3, OPA 2 gave 67% to 100% similar profiles. Moreover HGM 26 and HGM 38 share 100% similarity in RAPD profile as well as in dendrogram also. Therefore there similarity matrix value is 1

Fig 7.5 : Analysis of RAPD bands obtained by OPA 3



As shown in figure 7.5, the RAPD profile generated with primer OPA3 produced 25 bands of different molecular weight ranging from 93-1122 bp. Out of total 25, 23 bands were found to be polymorphic giving 92% polymorphism. All the 8 RAPD phenotypes obtained by OPA 3 were different. Hence OPA 3 has 100% differentiating power among the isolates tested. As seen in table 7.4, the lowest similarity obtained with

OPA 3 was 19% and least similar isolate was HGM 17 since it has given only 2 bands as against 9 to 19 bands given by other isolates. HGM 26 and HGM 30 gave 94% similar phenotype (fig. 7.6). Hence with OPA 3, 19% to 94% similar profiles were obtained.

Fig 7.6 : Dendrogram of isolates as obtained by OPA 3

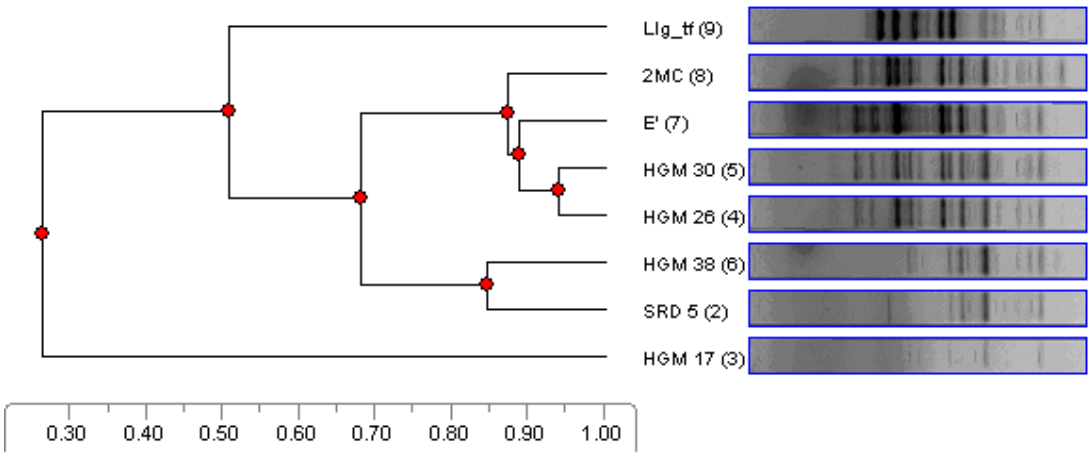
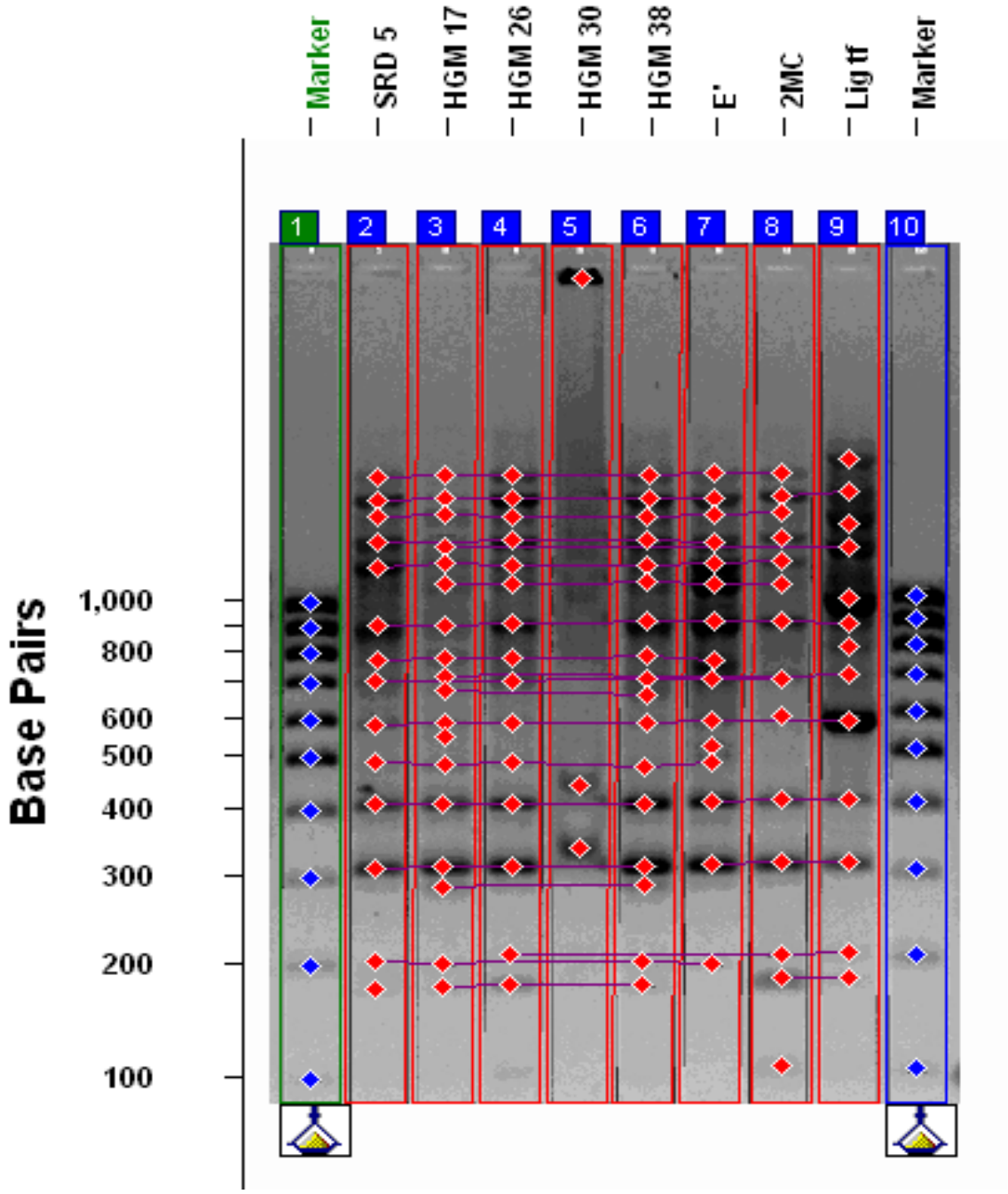


Table 7.4: Similarity matrix given by OPA 3

Lane	SRD 5	HGM 17	HGM 26	HGM 30	HGM 38	E'	2MC	Lig tf
SRD 5	1	0.36	0.67	0.62	0.84	0.57	0.64	0.48
HGM 17	0.36	1	0.24	0.21	0.33	0.19	0.19	0.29
HGM 26	0.67	0.24	1	0.94	0.8	0.88	0.88	0.52
HGM 30	0.62	0.21	0.94	1	0.74	0.89	0.89	0.55
HGM 38	0.84	0.33	0.8	0.74	1	0.69	0.69	0.45
E'	0.57	0.19	0.88	0.89	0.69	1	0.84	0.52
2MC	0.64	0.19	0.88	0.89	0.69	0.84	1	0.52
Lig tf	0.48	0.29	0.52	0.55	0.45	0.52	0.52	1

Fig 7.7 : Analysis of RAPD bands obtained by OPA 4



As shown in figure 7.7, the RAPD profile generated with primer OPA4 produced 37 bands of different molecular weight ranging from 157-1483 bp. All the 37 bands were found to be polymorphic giving 100% polymorphism. All the 8 RAPD phenotypes obtained by OPA 4 were also different. Hence OPA 4 has 100% differentiating power among the

isolates tested. With OPA 4, 0 to 97% similar profiles were obtained (Table 7.5). Most dissimilar isolate was HGM 30 since it gave only 3 bands and none of them matched with any band given by other isolates. As seen in fig 7.8, HGM 17 and HGM 38 gave 97% similar phenotype. Similarly HGM 26 and SRD 5 also gave 97% similarity.

Fig 7.8 : Dendrogram of isolates as obtained by OPA 4

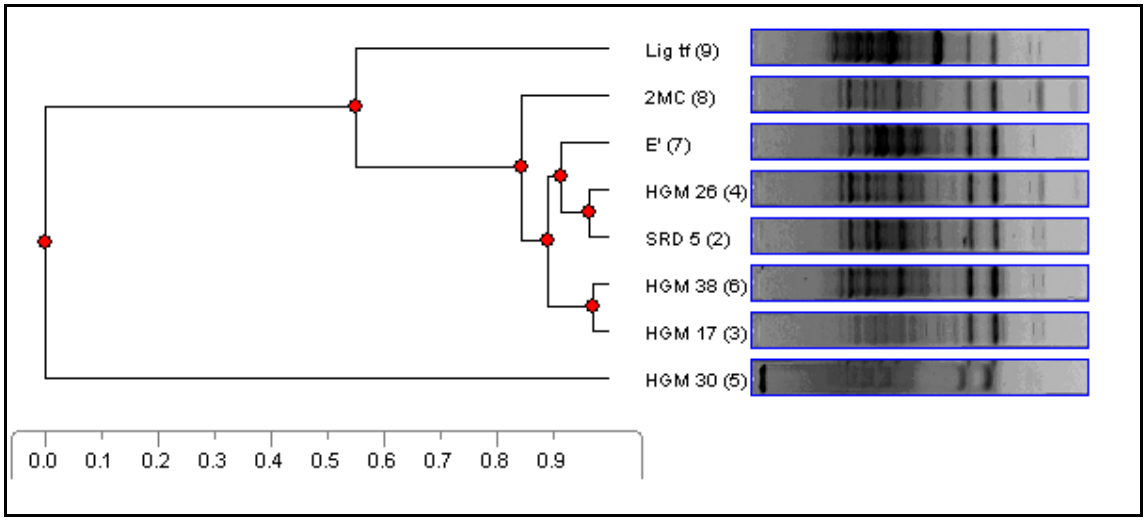
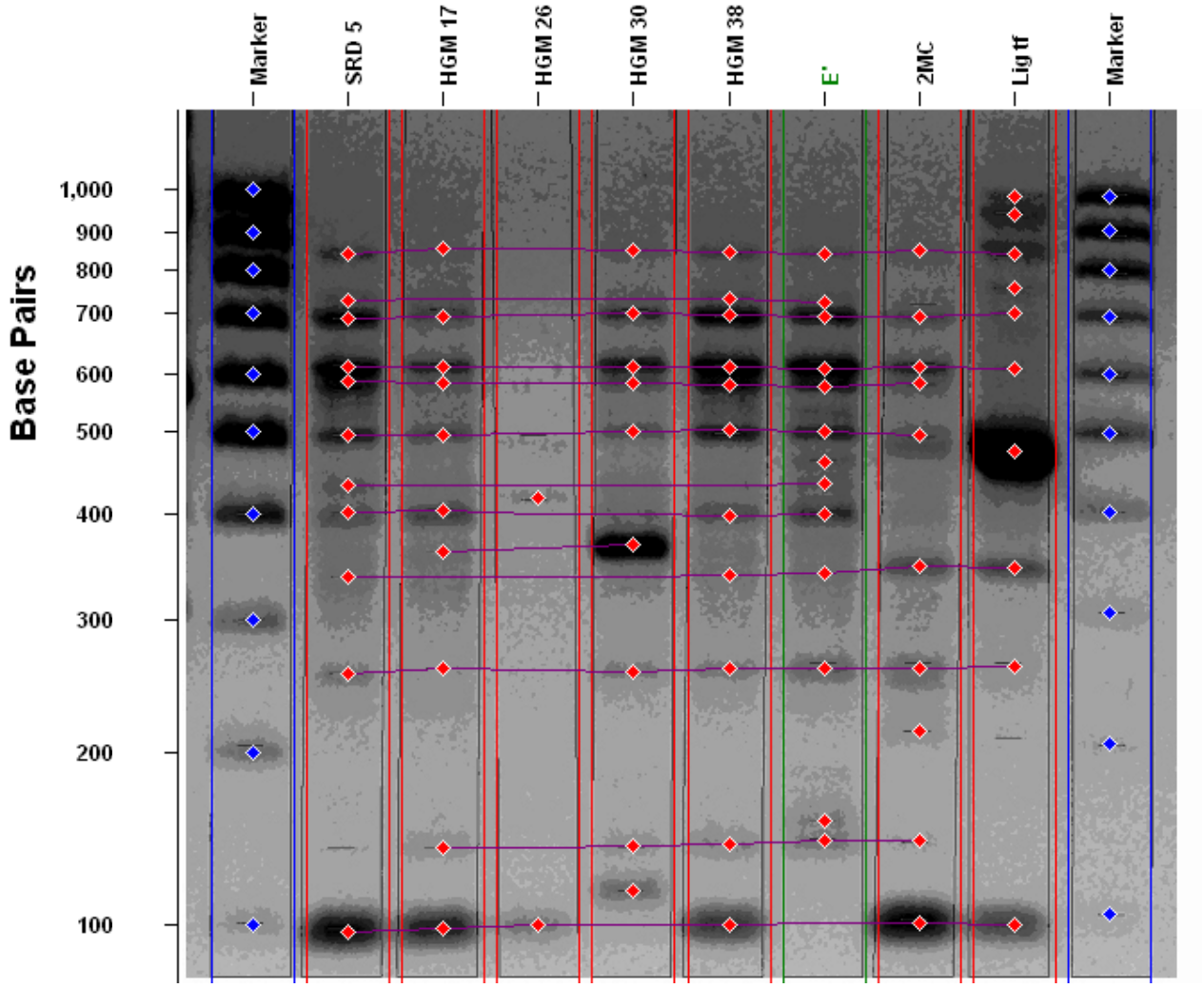


Table 7.5: Similarity matrix given by OPA 4

Lane	SRD 5	HGM 17	HGM 26	HGM 30	HGM 38	E'	2MC	Lig tf
SRD 5	1	0.88	0.97	0	0.9	0.9	0.86	0.59
HGM 17	0.88	1	0.91	0	0.97	0.85	0.81	0.52
HGM 26	0.97	0.91	1	0	0.94	0.93	0.9	0.57
HGM 30	0	0	0	1	0	0	0	0
HGM 38	0.9	0.97	0.94	0	1	0.88	0.84	0.53
E'	0.9	0.85	0.93	0	0.88	1	0.83	0.5
2MC	0.86	0.81	0.9	0	0.84	0.83	1	0.59
Lig tf	0.59	0.52	0.57	0	0.53	0.5	0.59	1

Fig 7.9 : Analysis of RAPD bands obtained by OPA 5



As shown in figure 7.9, the RAPD profile generated with primer OPA5 showed 22 bands of different molecular weight ranging from 23-877 bp. All the 22 bands were found to be polymorphic giving 100% polymorphism. All the 8 RAPD phenotypes obtained by OPA 5 were also different giving OPA 5, 100% differentiating power among the isolates tested. With OPA5, 0 to 91% similar profiles were obtained, which is a fairly wide range, confirming it as a good primer (Table 7.6). As seen in Figure 7.10 most dissimilar isolate was HGM 26 since it gave only 2 bands and one was completely polymorphic. Highest similar phenotype was shown by HGM 38 and SRD 5, which gave 91% similarity.

Fig 7.10 : Dendrogram of isolates as obtained by OPA 5

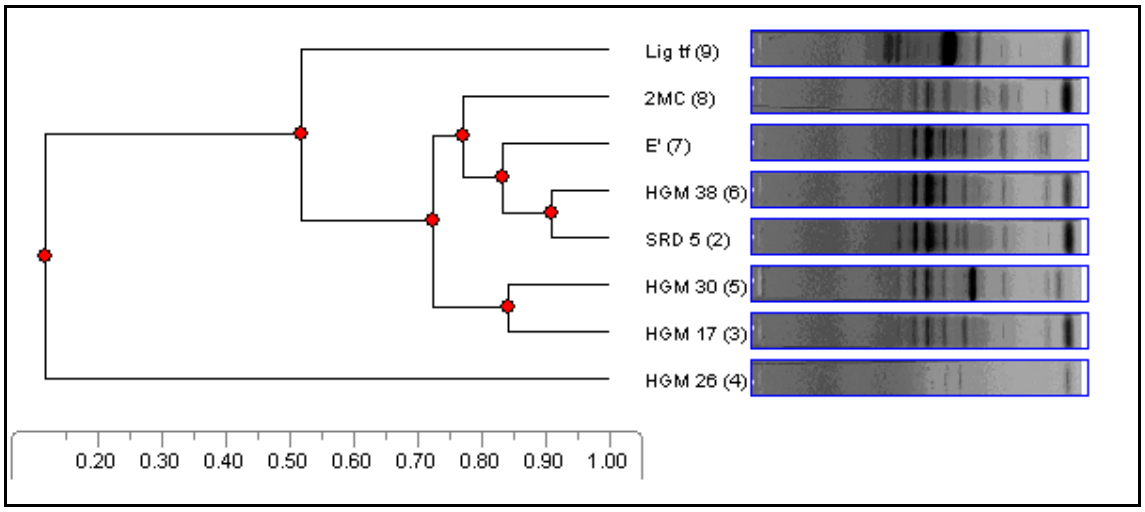
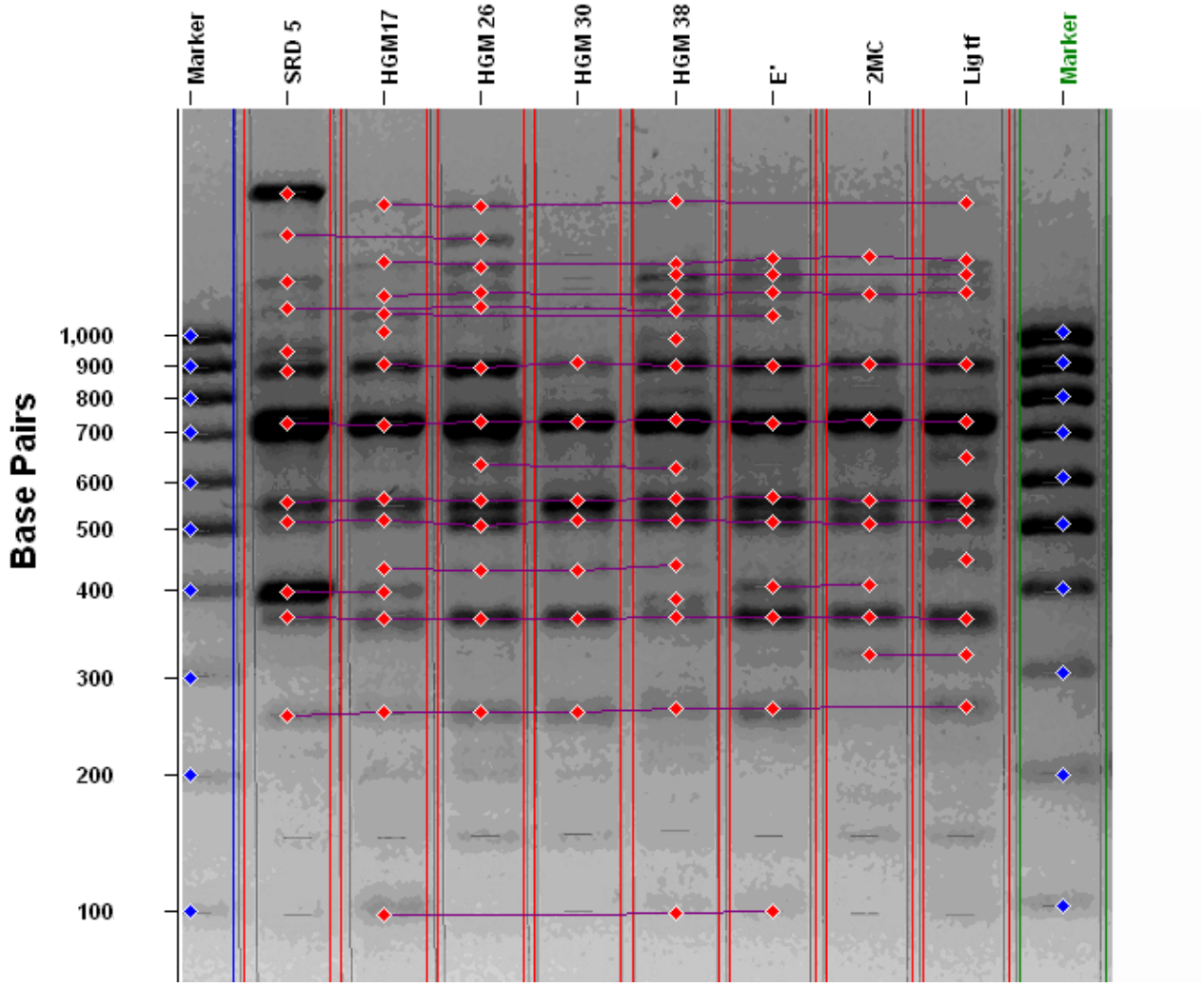


Table 7.6: Similarity matrix given by OPA 5

Lane	SRD 5	HGM 17	HGM 26	HGM 30	HGM 38	E'	2MC	Lig tf
SRD 5	1	0.76	0.15	0.6	0.91	0.83	0.76	0.57
HGM 17	0.76	1	0.17	0.84	0.86	0.7	0.8	0.5
HGM 26	0.15	0.17	1	0	0.15	0	0.17	0.17
HGM 30	0.6	0.84	0	1	0.7	0.64	0.74	0.42
HGM 38	0.91	0.86	0.15	0.7	1	0.83	0.86	0.57
E'	0.83	0.7	0	0.64	0.83	1	0.7	0.43
2MC	0.76	0.8	0.17	0.74	0.86	0.7	1	0.6
Lig tf	0.57	0.5	0.17	0.42	0.57	0.43	0.6	1

Fig 7.11 : Analysis of RAPD bands obtained by OPA 6



As shown in figure 7.11, the fragment profile generated from DNA sample of all 8 isolates with primer OPA 6 gave 29 bands out of which 25 bands were polymorphic. Hence, it gave 86% polymorphism. The bands ranged in size from 94-1465 base pairs. All the 8 RAPD phenotypes obtained were different, showing it to be good primer giving 100% differentiation. As seen in table 7.7, OPA 6 gave 38% to 77% similarity between isolates with isolate SRD 5 being the most dissimilar from all the isolates (Fig. 7.12).

Fig 7.12 : Dendrogram of isolates as obtained by OPA 6

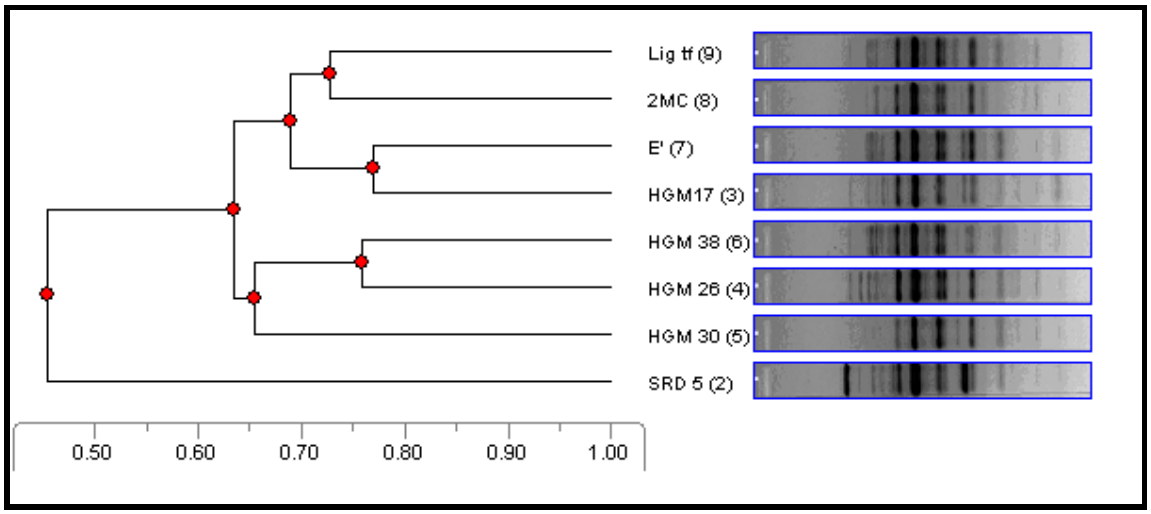
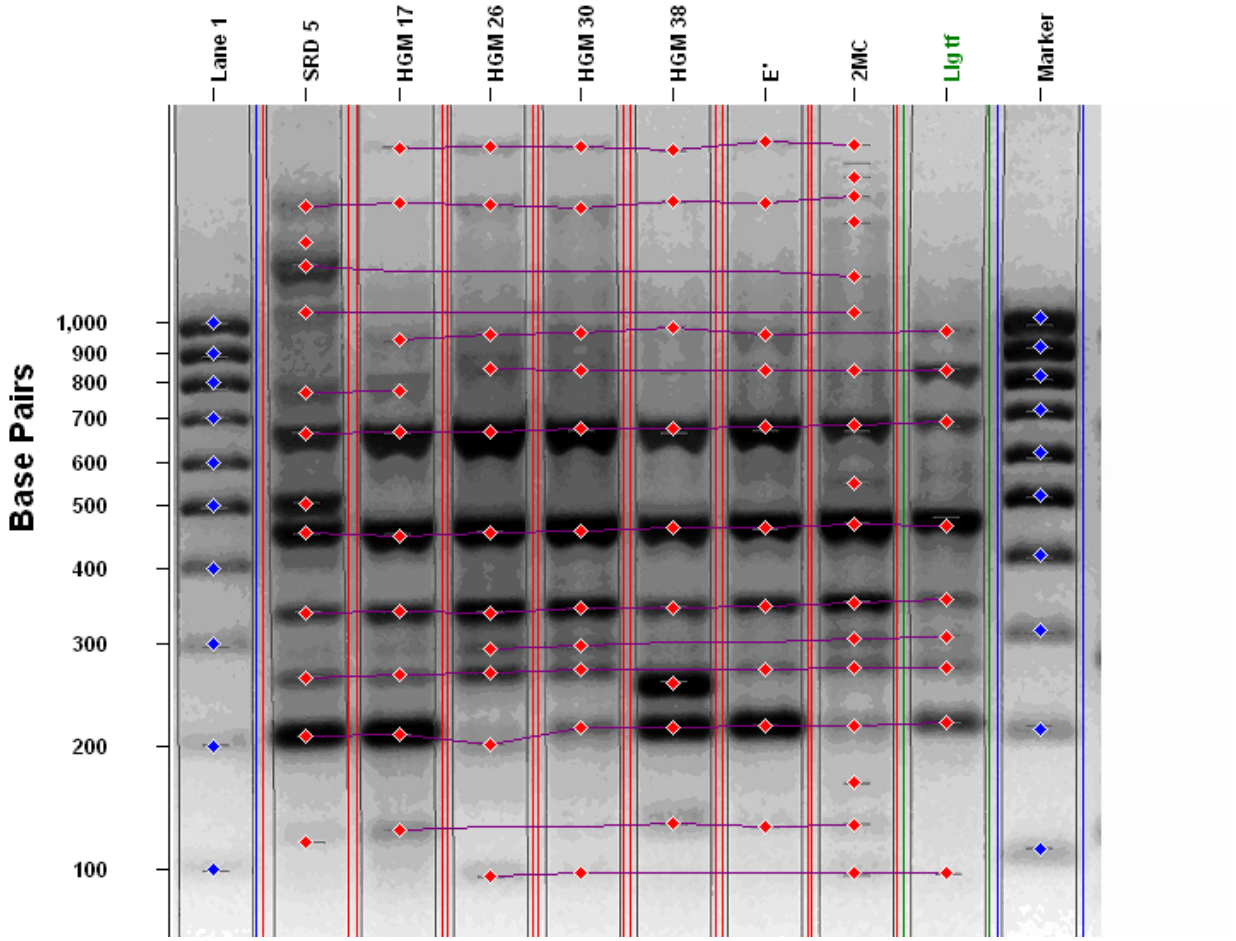


Table 7.7: Similarity matrix given by OPA 6

Lane	SRD 5	HGM 17	HGM 26	HGM 30	HGM 38	E'	2MC	Lig tf
SRD 5	1	0.46	0.56	0.53	0.43	0.42	0.38	0.4
HGM 17	0.46	1	0.67	0.67	0.73	0.77	0.61	0.67
HGM 26	0.56	0.67	1	0.7	0.76	0.56	0.55	0.62
HGM 30	0.53	0.67	0.7	1	0.61	0.63	0.63	0.6
HGM 38	0.43	0.73	0.76	0.61	1	0.71	0.56	0.69
E'	0.42	0.77	0.56	0.63	0.71	1	0.76	0.72
2MC	0.38	0.61	0.55	0.63	0.56	0.76	1	0.73
Lig tf	0.4	0.67	0.62	0.6	0.69	0.72	0.73	1

Fig 7.13 : Analysis of RAPD bands obtained by OPA 7



As shown in figure 7.13, the fragment profile generated from DNA sample of all 8 isolates with primer OPA 7 gave 23 bands, out of which 19 bands were polymorphic. Hence, it gave 83% polymorphism. The RAPD bands ranged from 33-1171 base pairs. Out of total 8 RAPD phenotypes, 7 phenotypes were different, giving the primer 87% differentiating power. As seen in fig. 7.14 and table 7.8, HGM 26 and HGM 30 gave 100% similar phenotype, while SRD 5 gave entirely different RAPD phenotype. With OPA 7, 48% to 100% similar profiles were obtained.

Fig 7.14 : Dendrogram of isolates as obtained by OPA 7

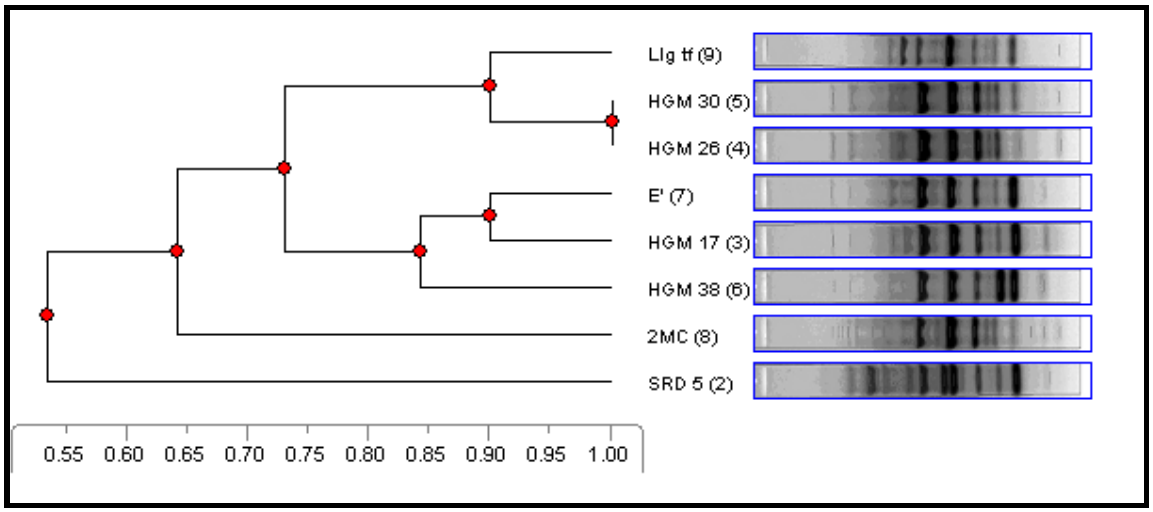
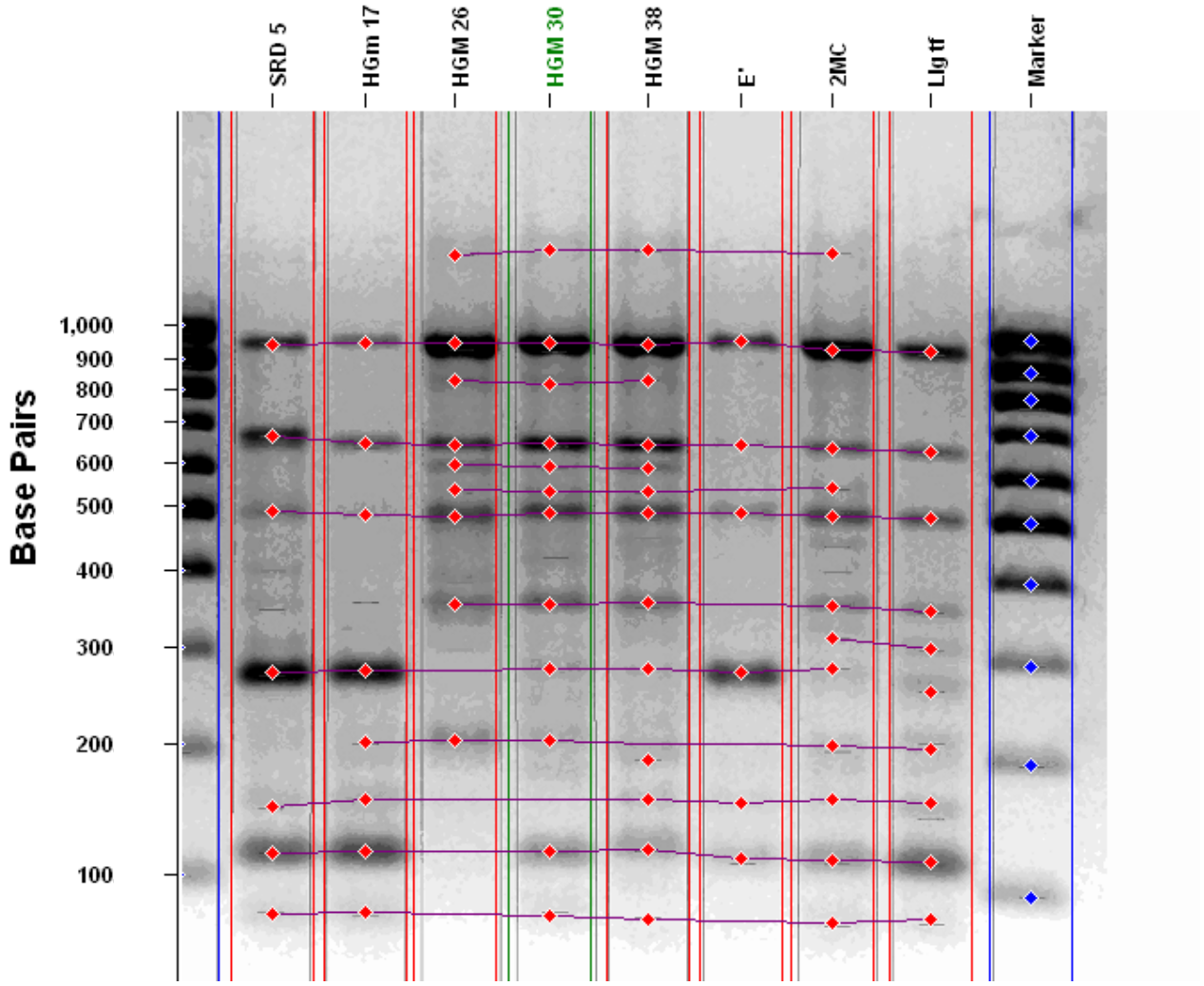


Table 7.8: Similarity matrix given by OPA 7

Lane	SRD 5	HGM 17	HGM 26	HGM 30	HGM 38	E'	2MC	Lig tf
SRD 5	1	0.64	0.52	0.52	0.48	0.55	0.55	0.48
HGM 17	0.64	1	0.76	0.76	0.84	0.9	0.59	0.63
HGM 26	0.52	0.76	1	1	0.7	0.86	0.71	0.9
HGM 30	0.52	0.76	1	1	0.7	0.86	0.71	0.9
HGM 38	0.48	0.84	0.7	0.7	1	0.84	0.54	0.56
E'	0.55	0.9	0.86	0.86	0.84	1	0.67	0.74
2MC	0.55	0.59	0.71	0.71	0.54	0.67	1	0.62
Lig tf	0.48	0.63	0.9	0.9	0.56	0.74	0.62	1

Fig 7.15 : Analysis of RAPD bands obtained by OPA 8



As shown in figure 7.15, the fragment profile generated by OPA 8 from all 8 isolates gave 16 bands, out of which 13 bands were polymorphic, showing 81% polymorphism. The bands ranged from 35 to 1013 bp. All the 8 RAPD phenotypes obtained were different, giving 100% differentiating power to primer. As described in table 7.9, primer OPA 8 gave RAPD profiles which were 38% to 93% similar. SRD 5 and HGM 17 showed 93% similarity. On the basis of fig. 7.16, all the 8 isolates were distributed in 2 broad groups, and one single isolate could not pointed out as different from rest.

Fig 7.16 : Dendrogram of isolates as obtained by OPA 8

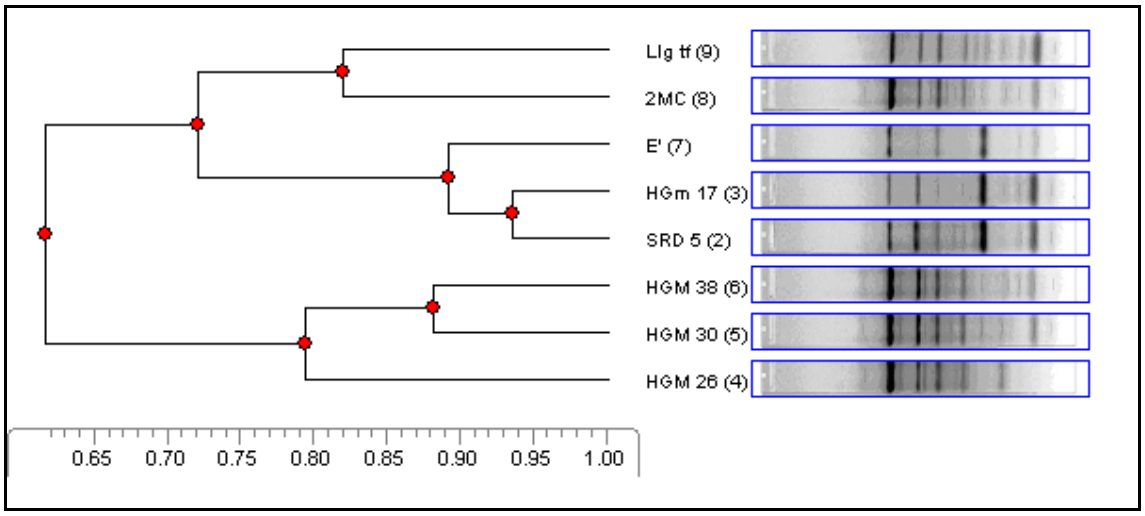
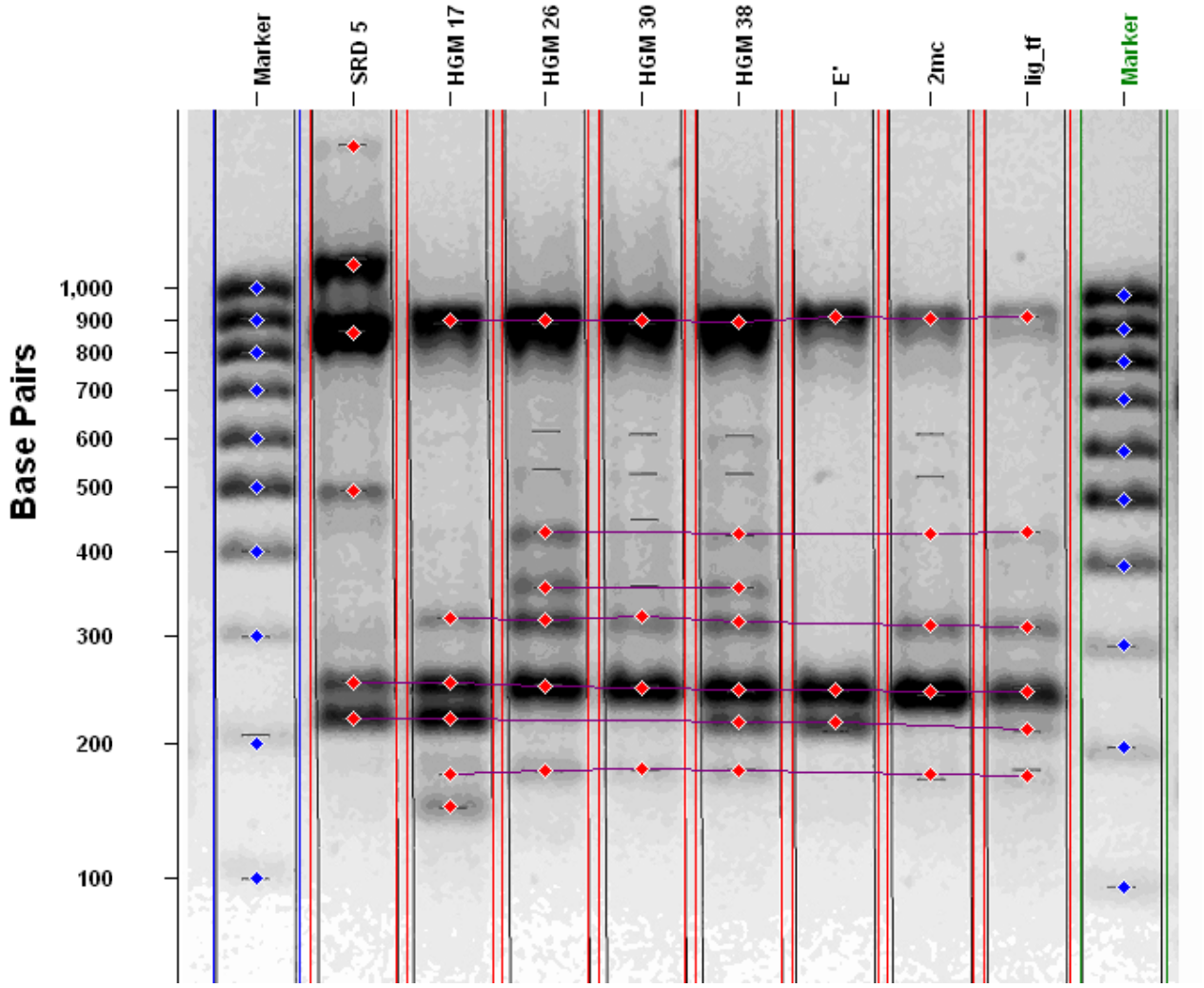


Table 7.9 Similarity matrix given by OPA 8

Lane	SRD 5	HGM 17	HGM 26	HGM 30	HGM 38	E'	2MC	Lig tf
SRD 5	1	0.93	0.38	0.63	0.7	0.92	0.74	0.71
HGM 17	0.93	1	0.47	0.7	0.67	0.86	0.8	0.78
HGM 26	0.38	0.47	1	0.86	0.73	0.4	0.67	0.53
HGM 30	0.63	0.7	0.86	1	0.88	0.56	0.83	0.64
HGM 38	0.7	0.67	0.73	0.88	1	0.63	0.8	0.61
E'	0.92	0.86	0.4	0.56	0.63	1	0.67	0.63
2MC	0.74	0.8	0.67	0.83	0.8	0.67	1	0.82
Lig tf	0.71	0.78	0.53	0.64	0.61	0.63	0.82	1

Fig 7.17 : Analysis of RAPD bands obtained by OPA 10



As shown in figure 7.17, the fragment profile generated from DNA sample of all 8 isolates with primer OPA 10 gave 12 bands, out of which 11 bands were polymorphic, showing 92% polymorphism. All the 8 RAPD phenotypes obtained were different from each other showing that primer OPA 10 had 100% differentiating power among all isolates. As seen in fig. 7.18 and table 7.10, SRD 5 gave entirely different RAPD phenotype, which was only 17% to 33% similar to other isolates. Lig tf and 2MC were 91% similar in their attributes while HGM 26 and HGM 38 were 92% similar as given in table 7.10. With OPA 10, 17% to 92% similar profiles were obtained.

Fig 7.18 : Dendrogram of isolates as obtained by OPA 10

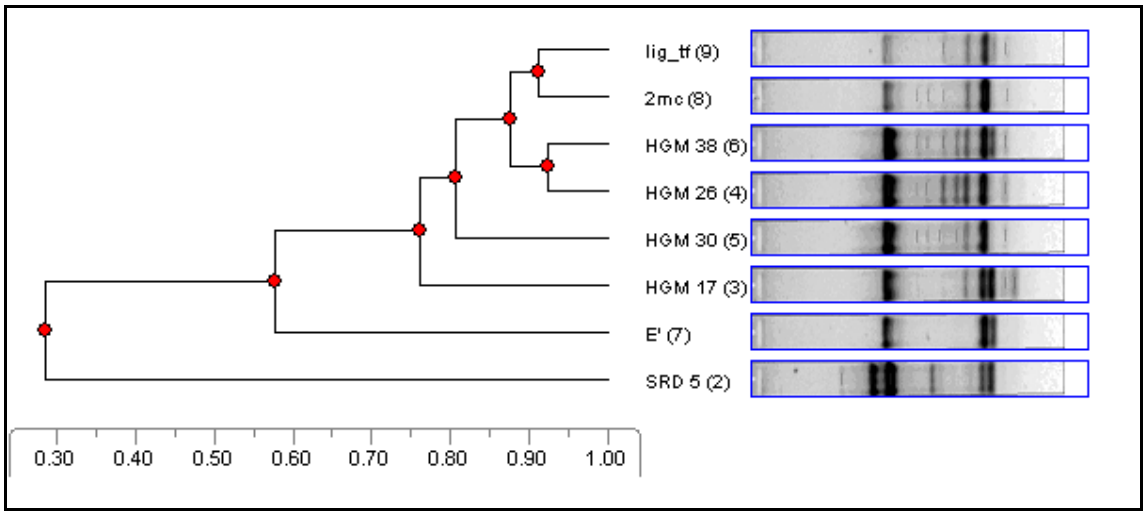
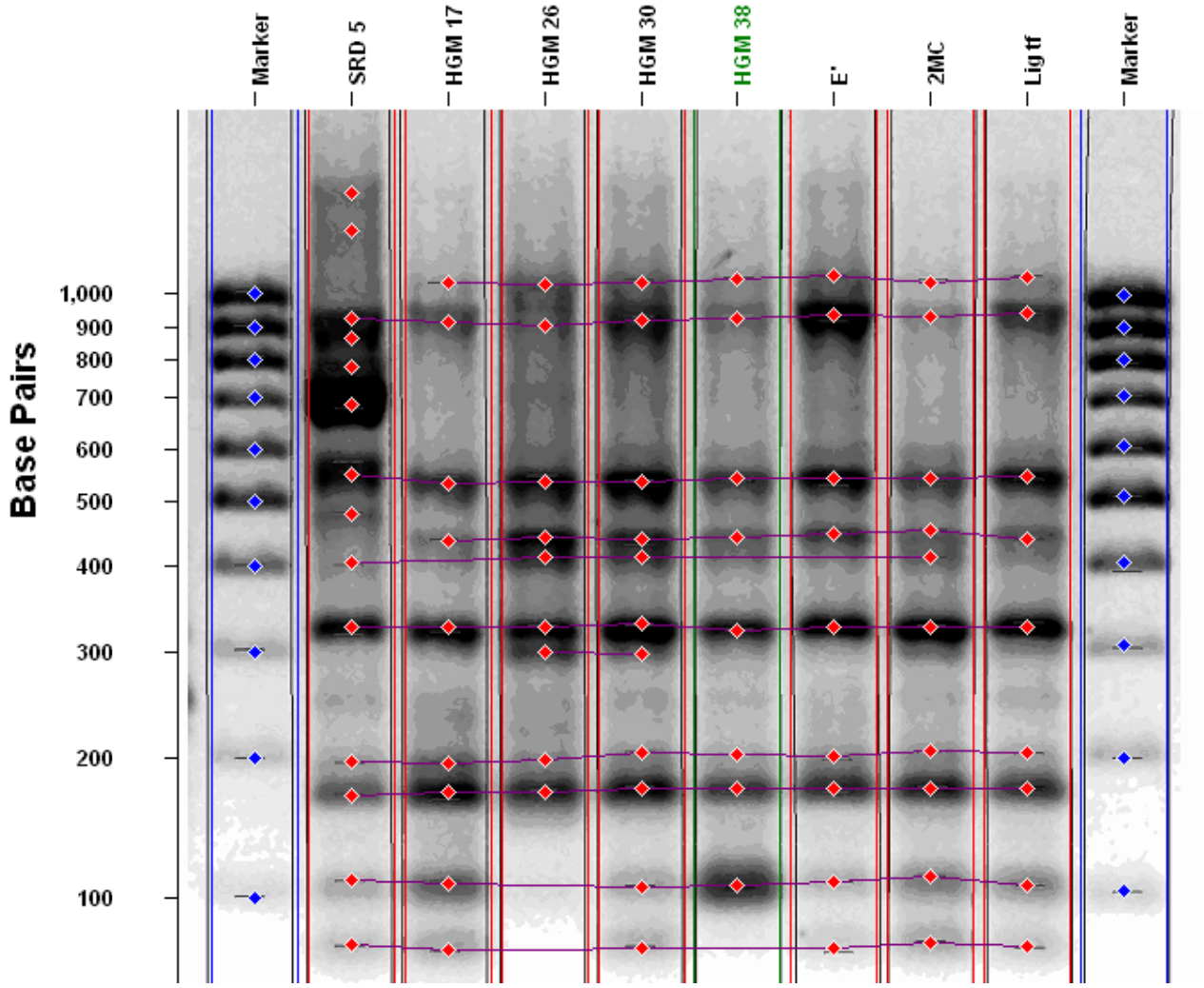


Table 7.10: Similarity matrix given by OPA 10

Lane	SRD 5	HGM 17	HGM 26	HGM 30	HGM 38	E'	2MC	Lig tf
SRD 5	1	0.33	0.17	0.2	0.31	0.44	0.18	0.33
HGM 17	0.33	1	0.67	0.8	0.77	0.67	0.73	0.83
HGM 26	0.17	0.67	1	0.8	0.92	0.44	0.91	0.83
HGM 30	0.2	0.8	0.8	1	0.73	0.57	0.89	0.8
HGM 38	0.31	0.77	0.92	0.73	1	0.6	0.83	0.92
E'	0.44	0.67	0.44	0.57	0.6	1	0.5	0.67
2MC	0.18	0.73	0.91	0.89	0.83	0.5	1	0.91
Lig tf	0.33	0.83	0.83	0.8	0.92	0.67	0.91	1

Fig 7.19 : Analysis of RAPD bands obtained by OPA 11



As shown in figure 7.19, the fragment profile generated from DNA sample of all 8 isolates with primer OPA 11 gave 17 bands, out of which 12 bands were polymorphic, giving 71% polymorphism. The RAPD bands ranged from 61-1295 base pairs. Out of total 8 RAPD phenotypes, 6 phenotypes were different, giving the primer 75% differentiating power. As seen in fig. 7.20 and table 7.11, Lig tf, E' and HGM 17 shared 100% similar phenotypes and grouped together, while SRD 5 was least similar to all other isolates. With OPA 11, 52% to 100% similar profiles were obtained. Most of the isolates were so similar that except SRD 5 all were more than 80% similar.

Fig 7.20 : Dendrogram of isolates as obtained by OPA 11

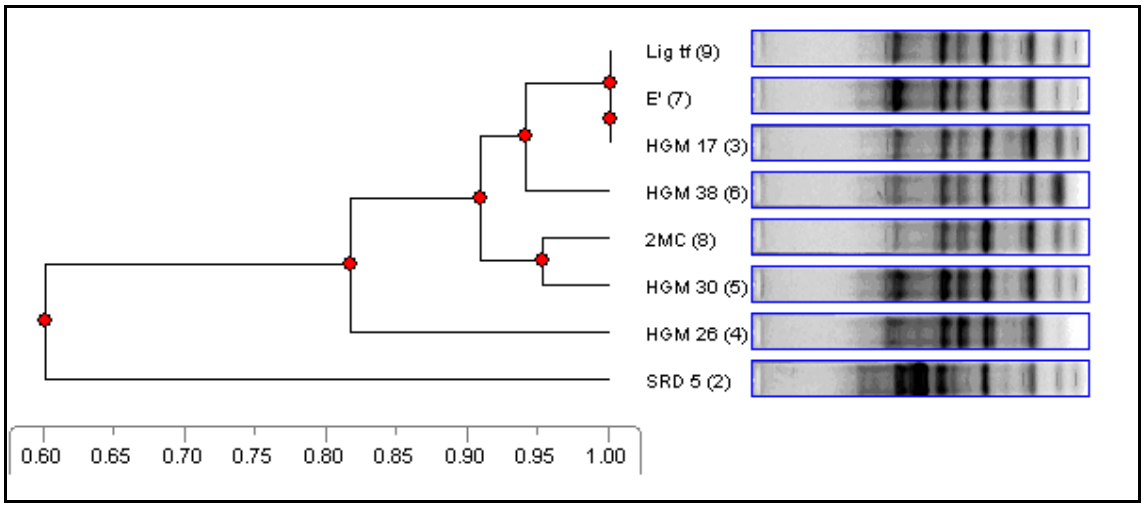
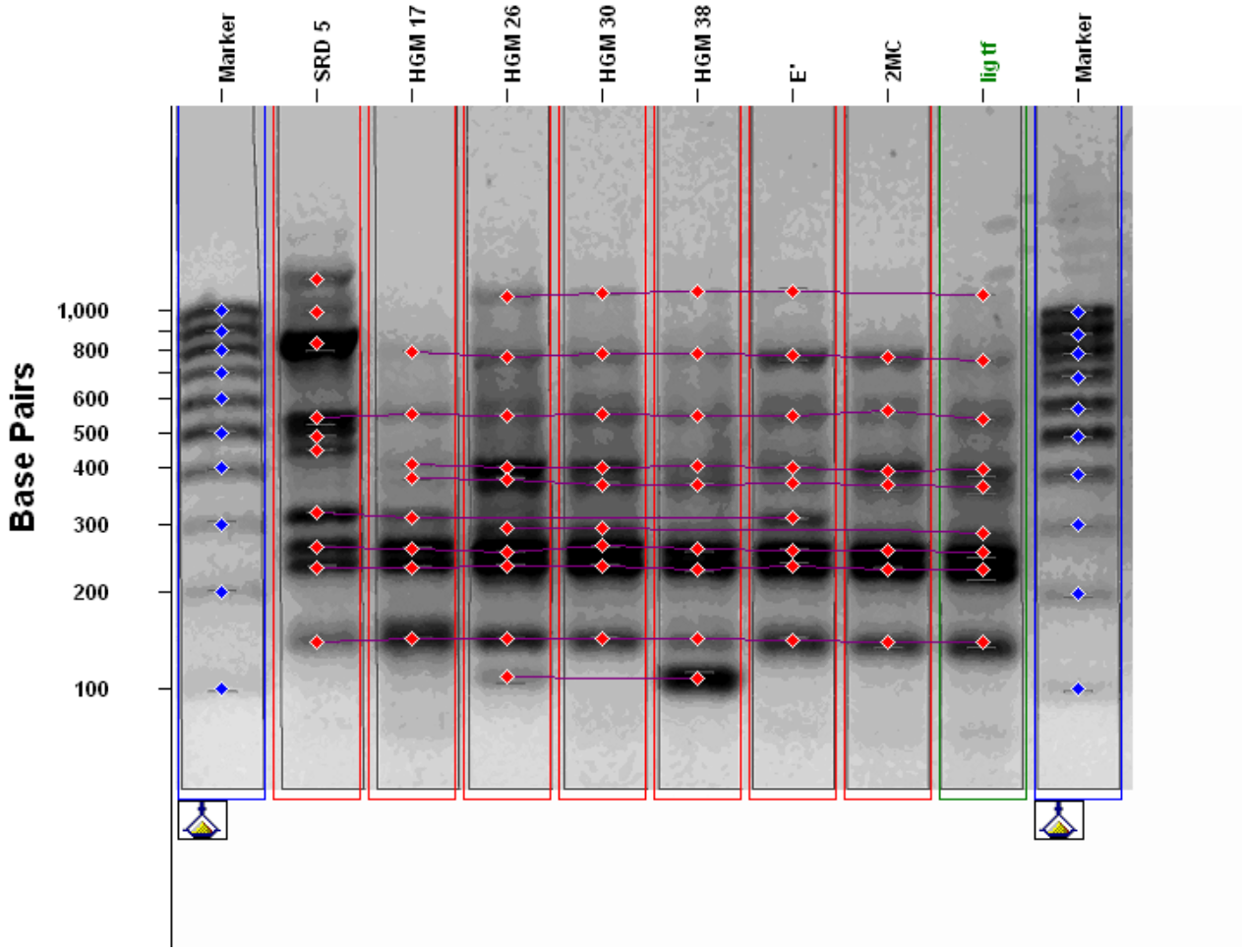


Table 7.11: Similarity matrix given by OPA 11

Lane	SRD 5	HGM 17	HGM 26	HGM 30	HGM 38	E'	2MC	Lig tf
SRD 5	1	0.61	0.52	0.64	0.55	0.61	0.67	0.61
HGM 17	0.61	1	0.78	0.9	0.94	1	0.95	1
HGM 26	0.52	0.78	1	0.9	0.82	0.78	0.84	0.78
HGM 30	0.64	0.9	0.9	1	0.84	0.9	0.95	0.9
HGM 38	0.55	0.94	0.82	0.84	1	0.94	0.89	0.94
E'	0.61	1	0.78	0.9	0.94	1	0.95	1
2MC	0.67	0.95	0.84	0.95	0.89	0.95	1	0.95
Lig tf	0.61	1	0.78	0.9	0.94	1	0.95	1

Fig 7.21 : Analysis of RAPD bands obtained by OPA 12



As shown in figure 7.21, the fragment profile generated from DNA sample of all 8 isolates with primer OPA 12 gave 16 bands, out of which 12 bands were polymorphic. Hence, it gave 75% polymorphism. The RAPD bands ranged from 110-1150 base pairs. Out of total 8 RAPD phenotypes, 7 phenotypes were different, giving the primer 87% differentiating power. As seen in fig. 7.22 and table 7.12, Lig Tf and HGM 30 gave 100% similar phenotype, while SRD 5 gave entirely different RAPD phenotype. With OPA 12, 40% to 100% similar profiles were obtained.

Fig 7.22 : Dendrogram of isolates as obtained by OPA 12

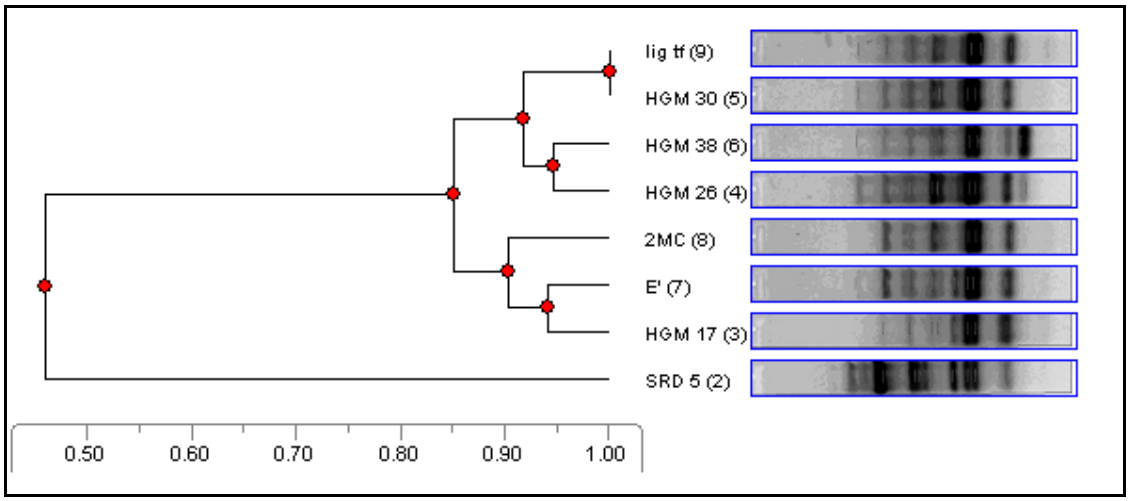
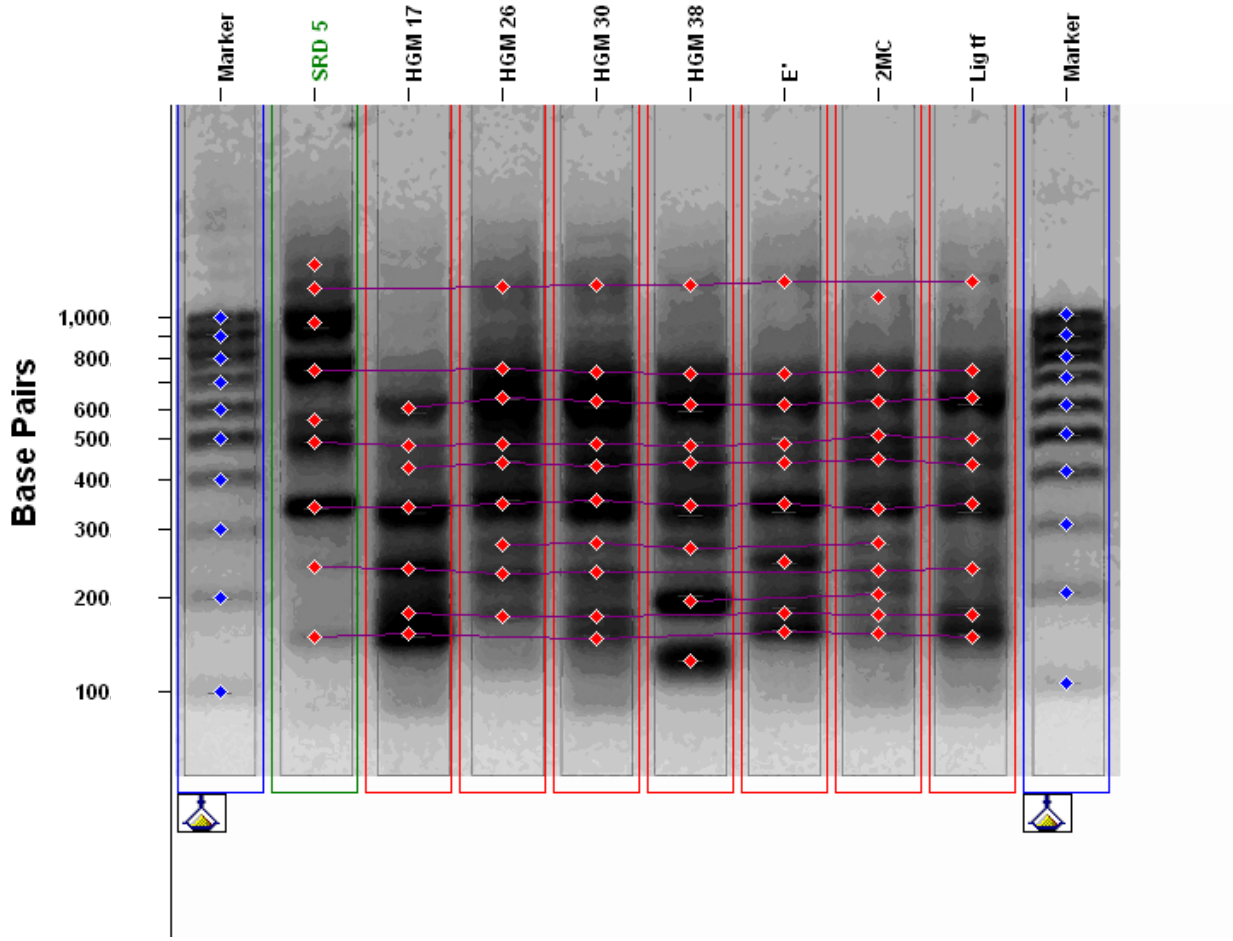


Table 7.12: Similarity matrix given by OPA 12

Lane	SRD 5	HGM 17	HGM 26	HGM 30	HGM 38	E'	2MC	Lig tf
SRD 5	1	0.56	0.4	0.42	0.42	0.53	0.47	0.42
HGM 17	0.56	1	0.78	0.82	0.82	0.94	0.93	0.82
HGM 26	0.4	0.78	1	0.95	0.95	0.84	0.82	0.95
HGM 30	0.42	0.82	0.95	1	0.89	0.89	0.88	1
HGM 38	0.42	0.82	0.95	0.89	1	0.89	0.88	0.89
E'	0.53	0.94	0.84	0.89	0.89	1	0.88	0.89
2MC	0.47	0.93	0.82	0.88	0.88	0.88	1	0.88
Lig tf	0.42	0.82	0.95	1	0.89	0.89	0.88	1

Fig 7.23 : Analysis of RAPD bands obtained by OPA 13



As shown in figure 7.23, the fragment profile generated from DNA sample of all 8 isolates with primer OPA 13 gave 17 bands, out of which 15 bands were polymorphic. The RAPD bands ranged from 126-1282 base pairs. All the 8 RAPD phenotypes were different. OPA 13 gave 44% to 95% similar profiles of the isolates. As seen in fig. 7.24 and table 7.13, HGM 26 and HGM 30 gave 95% similar phenotype, while SRD 5 gave entirely different RAPD phenotype.

Fig 7.24 : Dendrogram of isolates as obtained by OPA 13

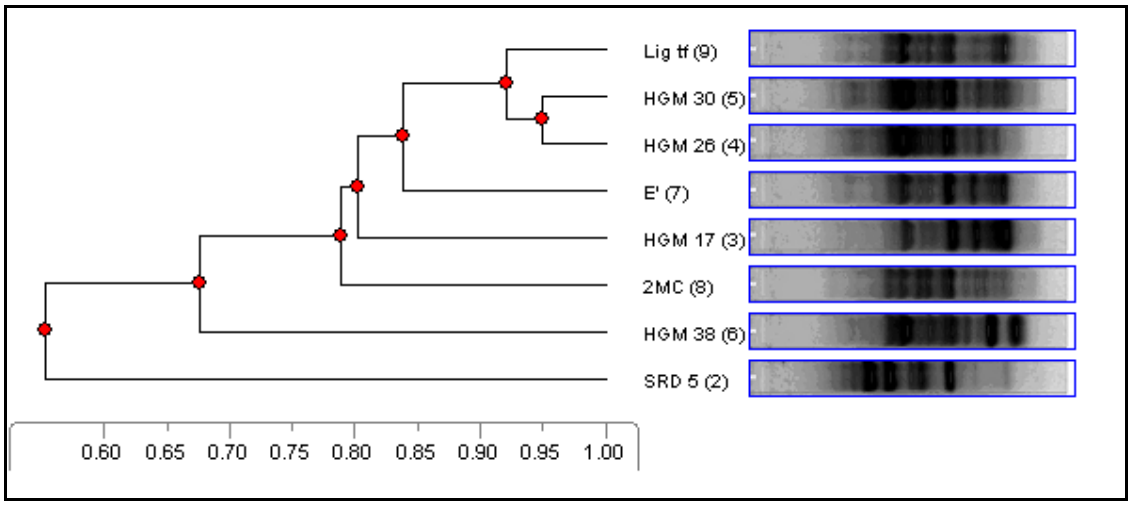
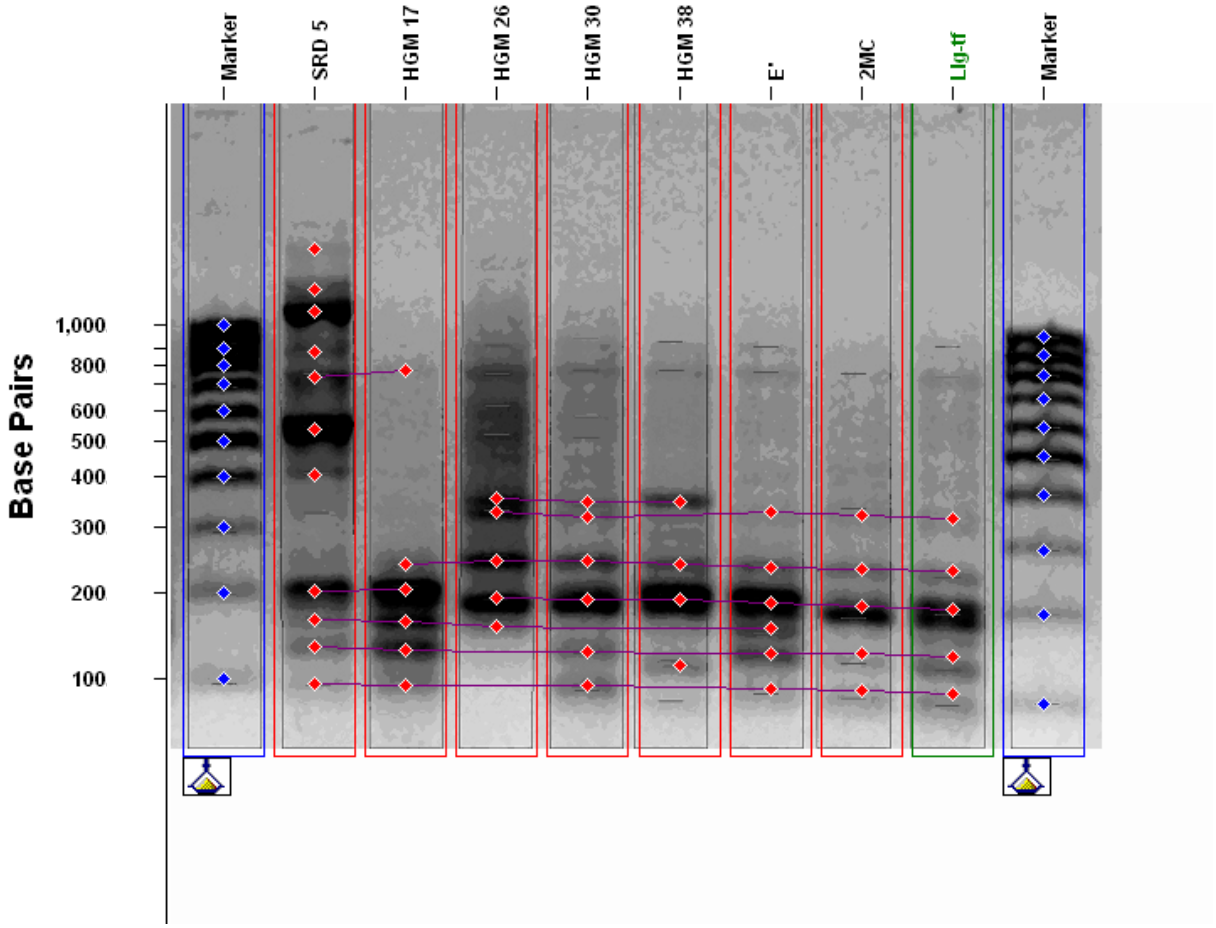


Table 7.13: Similarity matrix given by OPA 13

Lane	SRD 5	HGM 17	HGM 26	HGM 30	HGM 38	E'	2MC	Lig tf
SRD 5	1	0.5	0.56	0.63	0.44	0.56	0.5	0.67
HGM 17	0.5	1	0.75	0.82	0.5	0.75	0.78	0.88
HGM 26	0.56	0.75	1	0.95	0.78	0.78	0.8	0.89
HGM 30	0.63	0.82	0.95	1	0.74	0.84	0.86	0.95
HGM 38	0.44	0.5	0.78	0.74	1	0.67	0.7	0.67
E'	0.56	0.75	0.78	0.84	0.67	1	0.7	0.89
2MC	0.5	0.78	0.8	0.86	0.7	0.7	1	0.8
Lig tf	0.67	0.88	0.89	0.95	0.67	0.89	0.8	1

Fig 7.25 : Analysis of RAPD bands obtained by OPA 14



As shown in figure 7.25, the fragment profile generated from DNA sample of all 8 isolates with primer OPA 14 gave 16 bands, all of which were polymorphic, hence this primer gave 100% polymorphism of isolates. The RAPD bands ranged from 104-1354 base pairs. Out of total 8 RAPD phenotypes, 7 phenotypes were different, giving the primer 87% differentiating power. As seen in fig. 7.26, 0% to 100% similarity in profiles was obtained. Lig Tf and 2MC gave 100% similar phenotype, while SRD 5 and HGM 17 gave entirely different RAPD phenotype (Table 7.14).

Fig 7.26 : Dendrogram of isolates as obtained by OPA 14

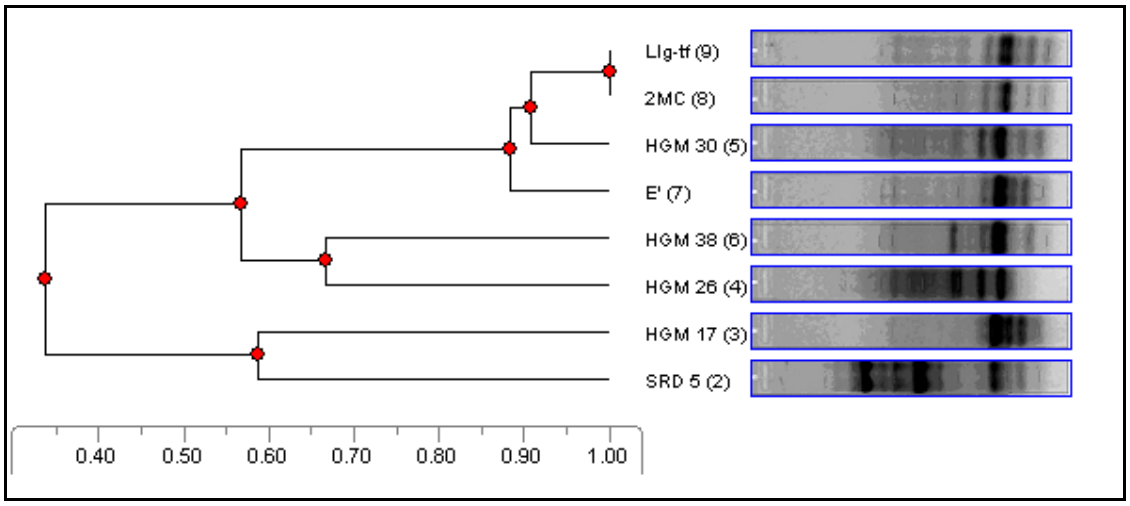
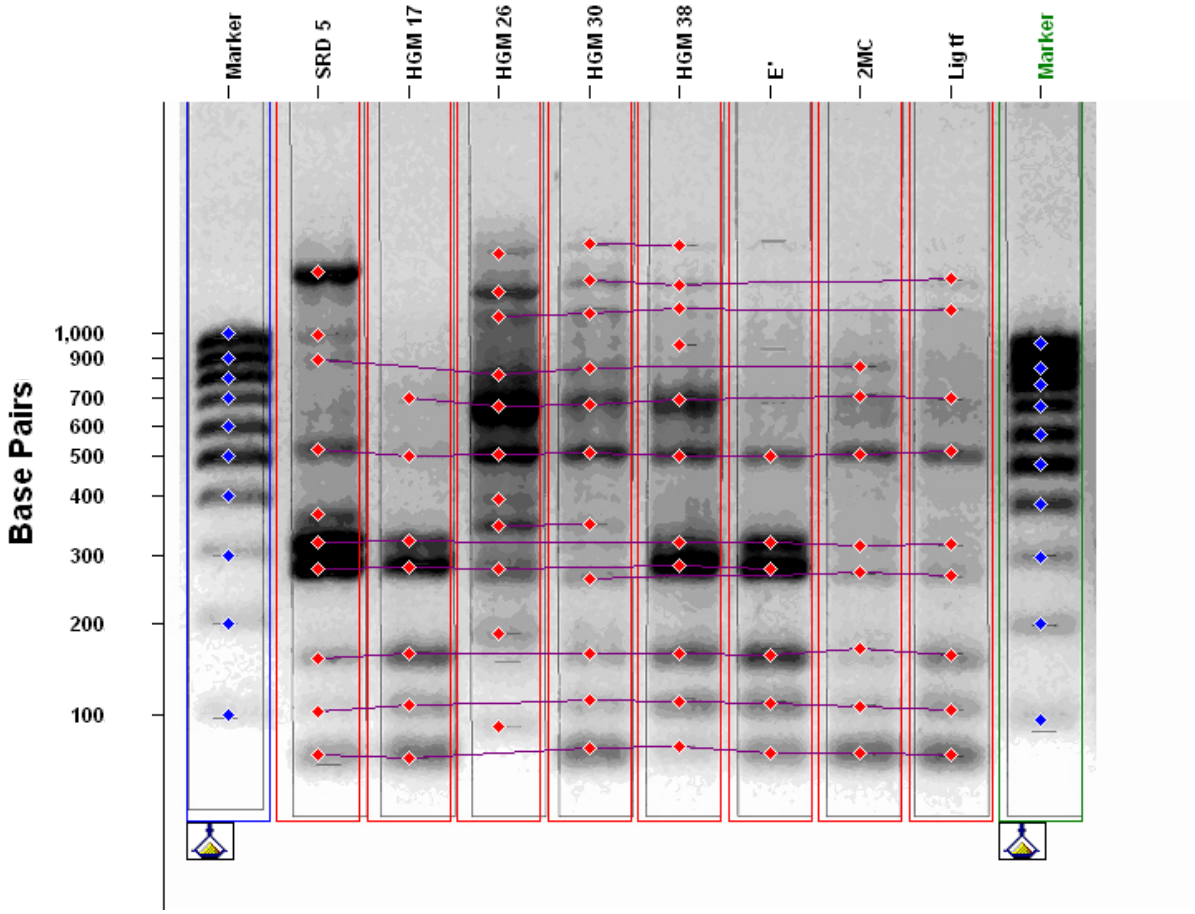


Table 7.14: Similarity matrix given by OPA 14

Lane	SRD 5	HGM 17	HGM 26	HGM 30	HGM 38	E'	2MC	Lig tf
SRD 5	1	0.59	0.13	0.24	0	0.35	0.25	0.25
HGM 17	0.59	1	0.36	0.5	0.2	0.67	0.55	0.55
HGM 26	0.13	0.36	1	0.73	0.67	0.73	0.6	0.6
HGM 30	0.24	0.5	0.73	1	0.6	0.83	0.91	0.91
HGM 38	0	0.2	0.67	0.6	1	0.4	0.44	0.44
E'	0.35	0.67	0.73	0.83	0.4	1	0.91	0.91
2MC	0.25	0.55	0.6	0.91	0.44	0.91	1	1
Lig tf	0.25	0.55	0.6	0.91	0.44	0.91	1	1

Fig 7.27 : Analysis of RAPD bands obtained by OPA 15



As shown in figure 7.27, the fragment profile generated from DNA sample of all 8 isolates with primer OPA 15 gave 22 bands, out of which 21 bands were polymorphic and the primer could give 95% polymorphism. The RAPD bands ranged from 62-1377 base pairs. Out of total 8 RAPD phenotypes all were different, giving the primer 100% differentiating power. As seen in fig. 7.28 and table 7.15, HGM 17 and E' gave 92% similar phenotype, while HGM 26 gave entirely different RAPD phenotype. With OPA 15, 24% to 92% similar profiles were obtained.

Fig 7.28 : Dendrogram of isolates as obtained by OPA 15

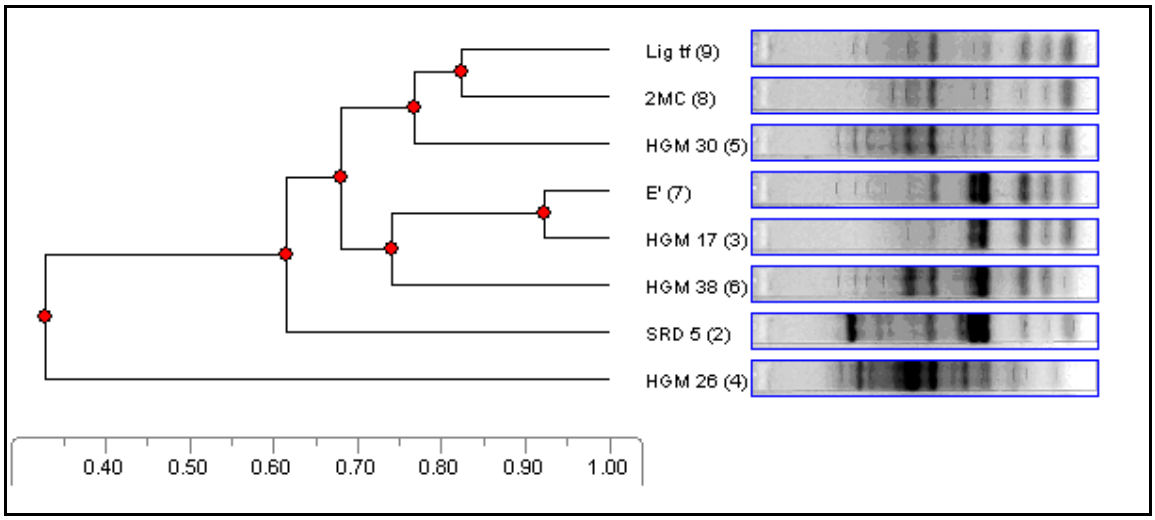
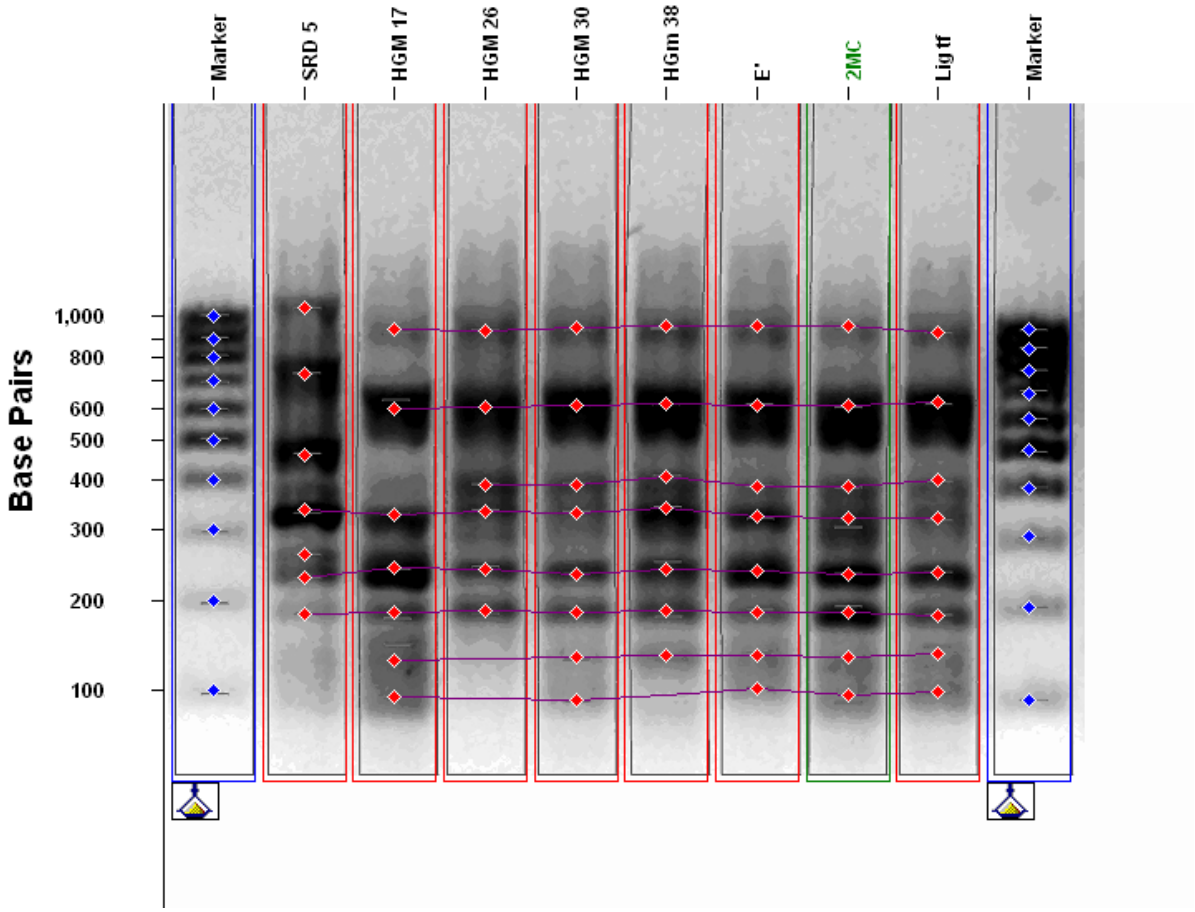


Table 7.15: Similarity matrix given by OPA 15

Lane	SRD 5	HGM 17	HGM 26	HGM 30	HGM 38	E'	2MC	Lig tf
SRD 5	1	0.71	0.29	0.48	0.57	0.75	0.67	0.53
HGM 17	0.71	1	0.33	0.56	0.78	0.92	0.8	0.75
HGM 26	0.29	0.33	1	0.45	0.36	0.24	0.32	0.3
HGM 30	0.48	0.56	0.45	1	0.73	0.47	0.74	0.8
HGM 38	0.57	0.78	0.36	0.73	1	0.71	0.63	0.8
E'	0.75	0.92	0.24	0.47	0.71	1	0.71	0.67
2MC	0.67	0.8	0.32	0.74	0.63	0.71	1	0.82
Lig tf	0.53	0.75	0.3	0.8	0.8	0.67	0.82	1

Fig 7.29 : Analysis of RAPD bands obtained by OPA 16



As shown in figure 7.29, the fragment profile generated from DNA sample of all 8 isolates with primer OPA 16 gave 12 bands, out of which 9 bands were polymorphic. Hence, 75% polymorphism was observed. The RAPD bands ranged from 102-1043 base pairs. Out of total 8 RAPD phenotypes, 5 phenotypes were different, giving the primer 62% differentiating power. As seen in fig. 7.30, four isolates – Lig Tf, HGM 30, E' and 2MC gave 100% similar phenotype, while SRD 5 was different from all other isolates. With OPA 16, 40% to 100% similar profiles were obtained (Table 7.16).

Fig 7.30 : Dendrogram of isolates as obtained by OPA 16

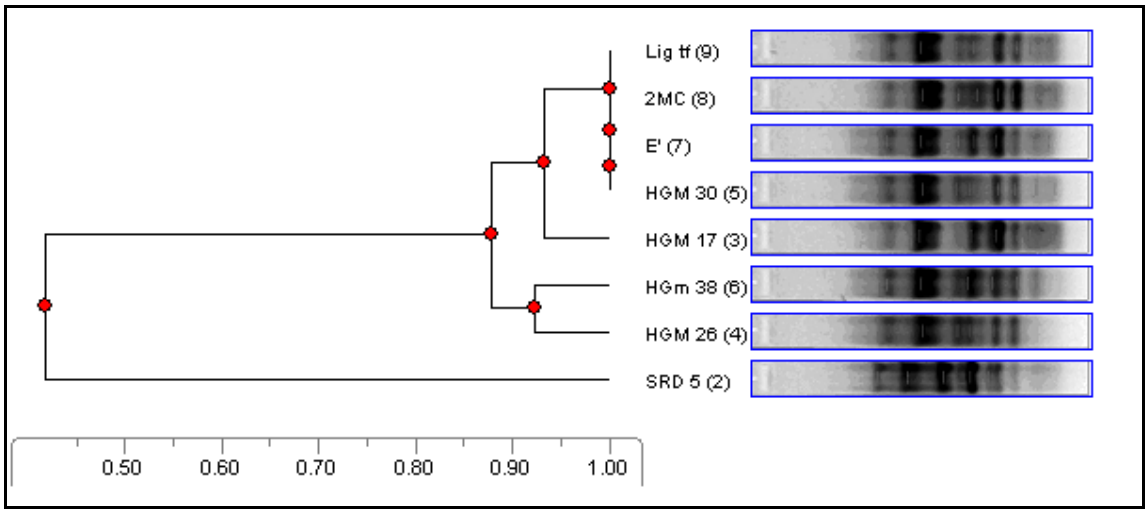
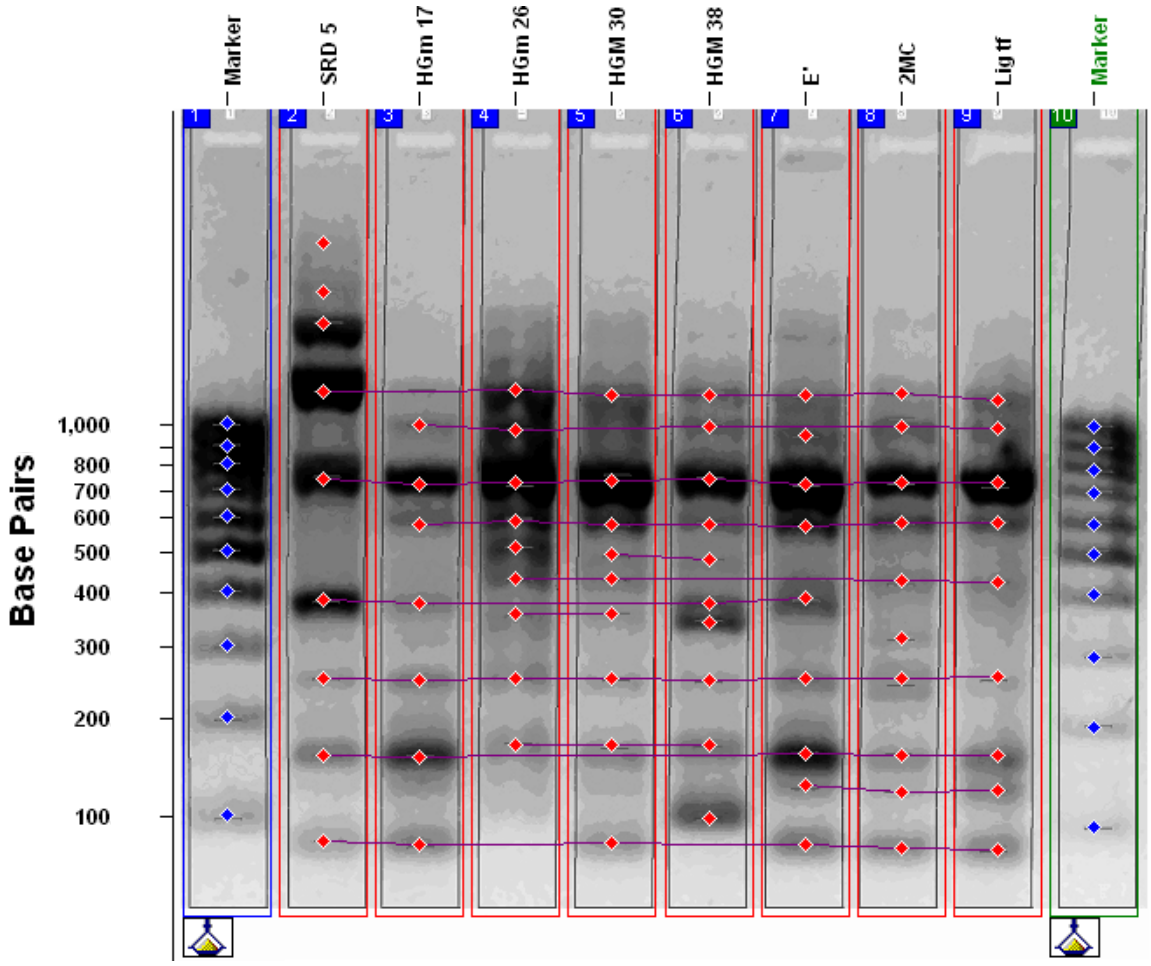


Table 7.16: Similarity matrix given by OPA 16

Lane	SRD 5	HGM 17	HGM 26	HGM 30	HGM 38	E'	2MC	Lig tf
SRD 5	1	0.43	0.46	0.4	0.43	0.4	0.4	0.4
HGM 17	0.43	1	0.77	0.93	0.86	0.93	0.93	0.93
HGM 26	0.46	0.77	1	0.86	0.92	0.86	0.86	0.86
HGM 30	0.4	0.93	0.86	1	0.93	1	1	1
HGM 38	0.43	0.86	0.92	0.93	1	0.93	0.93	0.93
E'	0.4	0.93	0.86	1	0.93	1	1	1
2MC	0.4	0.93	0.86	1	0.93	1	1	1
Lig tf	0.4	0.93	0.86	1	0.93	1	1	1

Fig 7.31 : Analysis of RAPD bands obtained by OPA 17



As shown in figure 7.31, the fragment profile generated from DNA sample of all 8 isolates with primer OPA 17 gave 21 bands, out of which 19 bands were polymorphic. Hence the primer gave 90% polymorphism. The RAPD bands ranged from 76-1792 base pairs. All the 8 RAPD phenotypes obtained were different, giving the primer 100% differentiating power. As seen in fig. 7.32, Lig Tf and 2MC gave 95% similar phenotype. With OPA 17, 33% to 95% similar profiles were obtained (table 7.17).

Fig 7.32 : Dendrogram of isolates as obtained by OPA 17

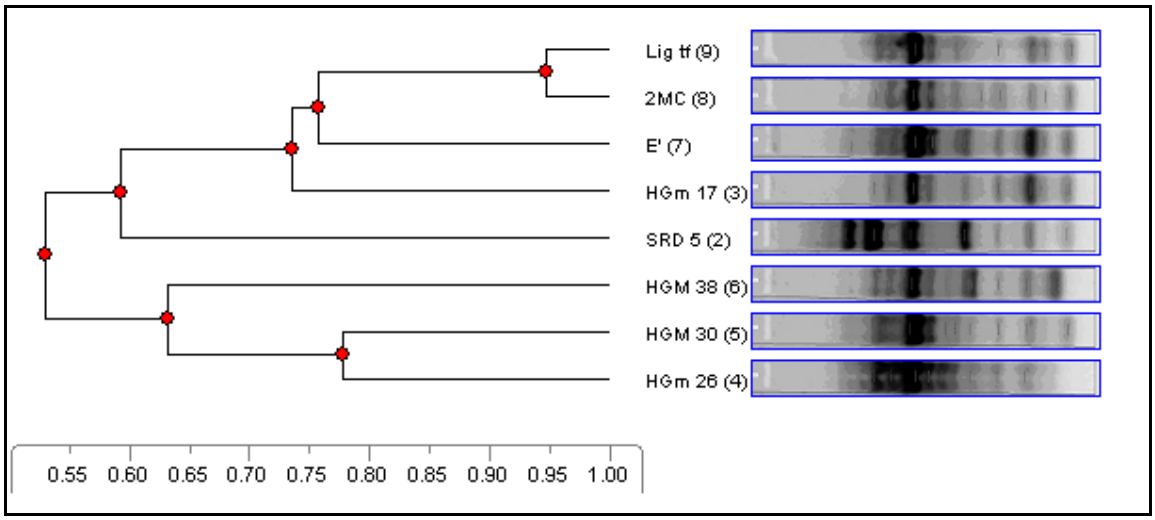
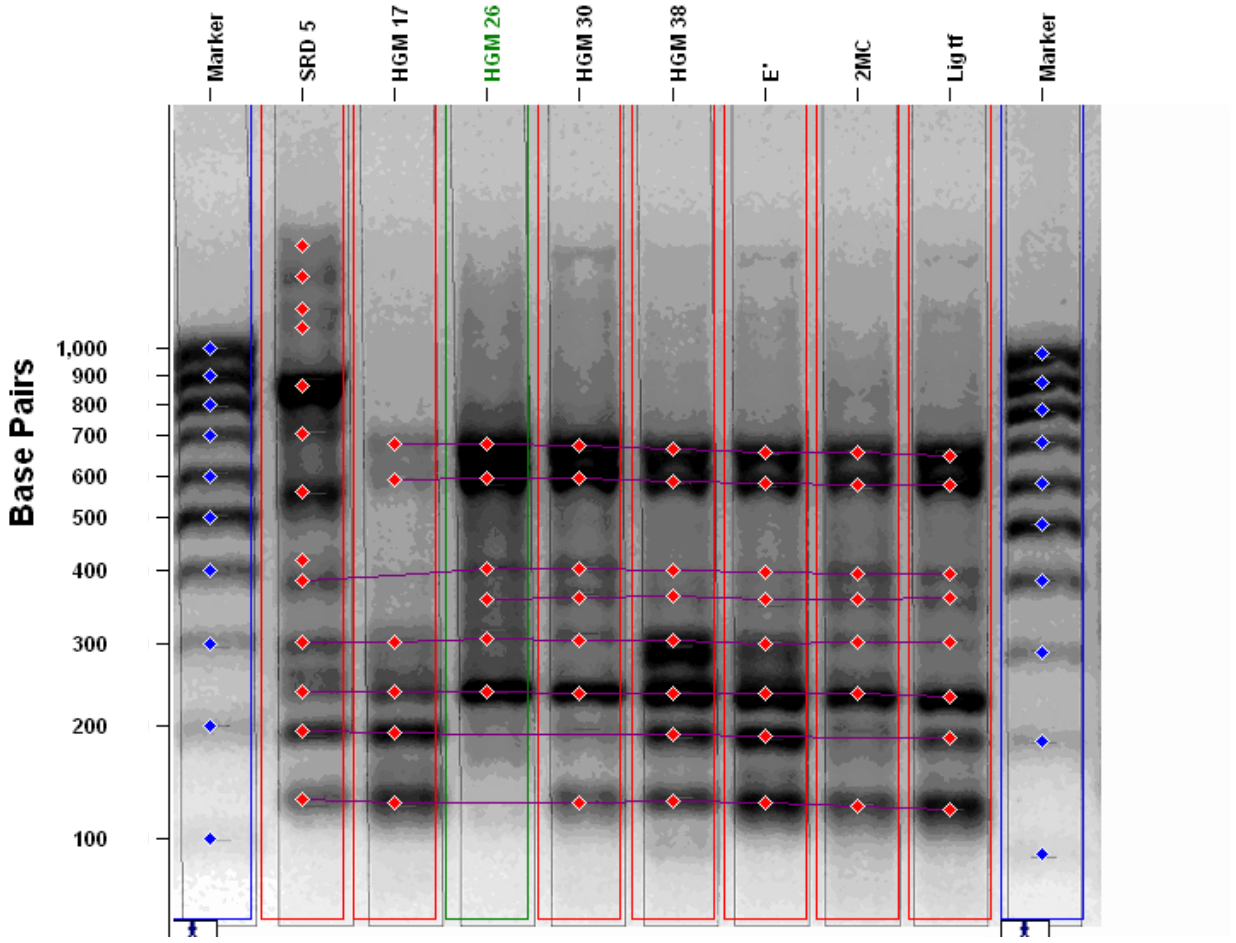


Table 7.17: Similarity matrix given by OPA 17

Lane	SRD 5	HGM 17	HGM 26	HGM 30	HGM 38	E'	2MC	Lig tf
SRD 5	1	0.63	0.33	0.44	0.42	0.67	0.53	0.56
HGM 17	0.63	1	0.5	0.5	0.59	0.75	0.71	0.75
HGM 26	0.33	0.5	1	0.78	0.63	0.44	0.63	0.67
HGM 30	0.44	0.5	0.78	1	0.63	0.56	0.63	0.67
HGM 38	0.42	0.59	0.63	0.63	1	0.53	0.5	0.53
E'	0.67	0.75	0.44	0.56	0.53	1	0.74	0.78
2MC	0.53	0.71	0.63	0.63	0.5	0.74	1	0.95
Lig tf	0.56	0.75	0.67	0.67	0.53	0.78	0.95	1

Fig 7.33 : Analysis of RAPD bands obtained by OPA 18



As shown in figure 7.33, the fragment profile generated from DNA sample of all 8 isolates with primer OPA 18 gave 16 bands, out of which 14 bands were polymorphic. The primer showed 87% polymorphism. The RAPD bands ranged from 136-1375 base pairs. Out of total 8 RAPD phenotypes, 5 phenotypes were different, giving the primer 62% differentiating power. As seen in fig. 7.34, three isolates – Lig Tf, HGM 38 and E' have shown 100% similar phenotype and similarly HGM 30 and 2MC have also shown 100% similarity in their attributes. SRD 5 was different from all other isolates. With OPA 18, 32% to 100% similar profiles were obtained (Table 7.18).

Fig 7.34 : Dendrogram of isolates as obtained by OPA 18

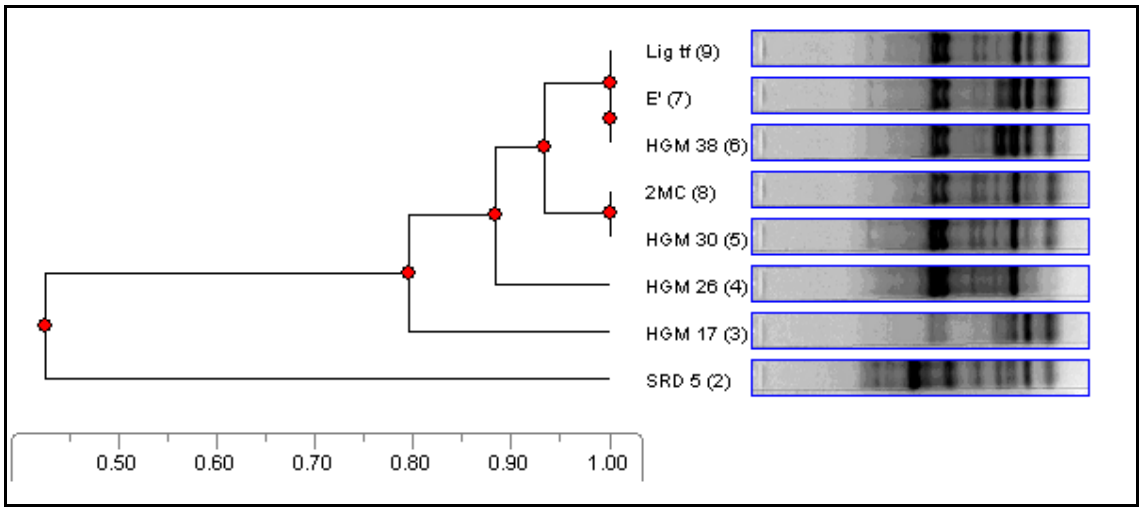
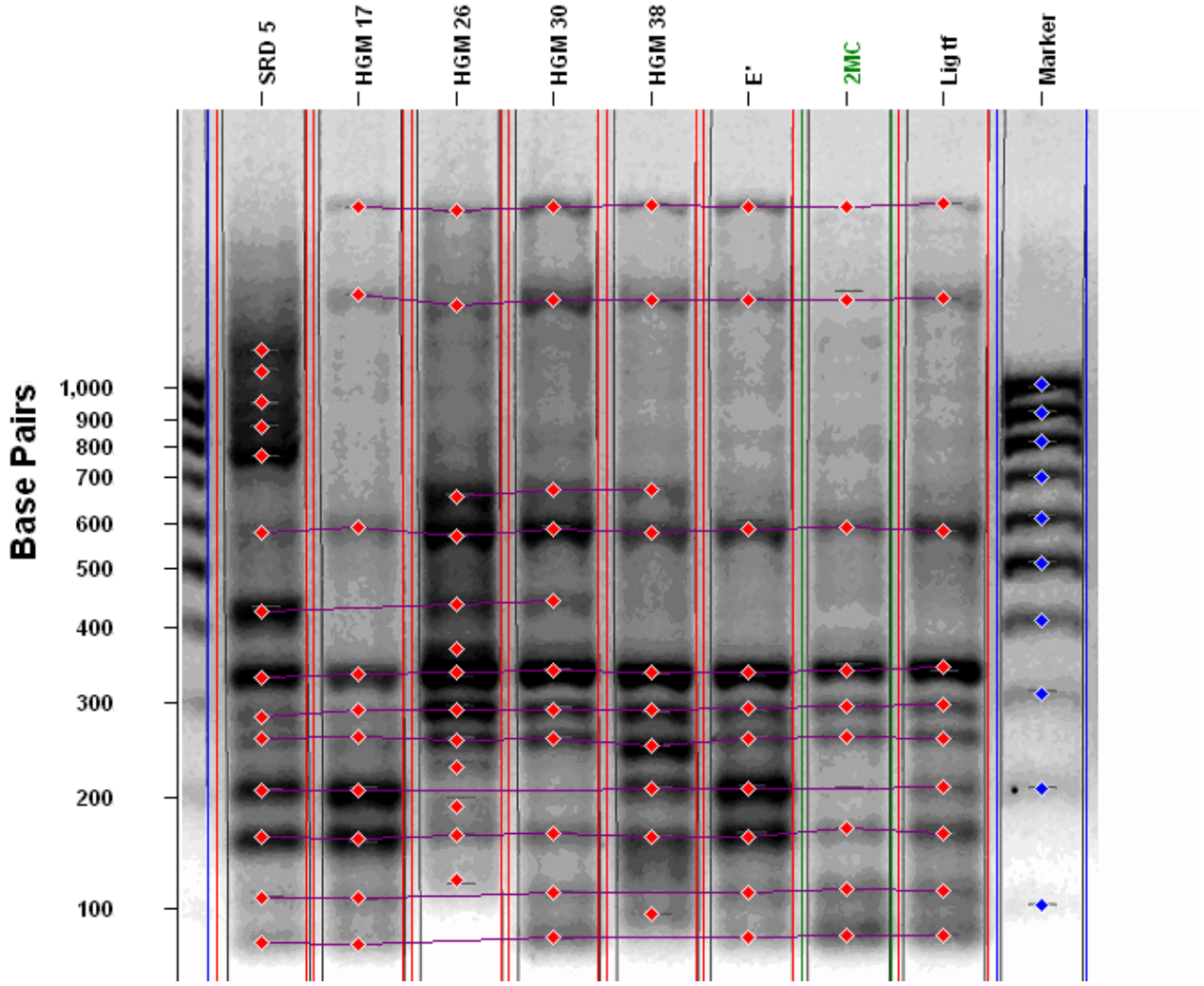


Table 7.18: Similarity matrix given by OPA 18

Lane	SRD 5	HGM 17	HGM 26	HGM 30	HGM 38	E'	2MC	Lig tf
SRD 5	1	0.42	0.32	0.4	0.48	0.48	0.4	0.48
HGM 17	0.42	1	0.67	0.77	0.86	0.86	0.77	0.86
HGM 26	0.32	0.67	1	0.92	0.86	0.86	0.92	0.86
HGM 30	0.4	0.77	0.92	1	0.93	0.93	1	0.93
HGM 38	0.48	0.86	0.86	0.93	1	1	0.93	1
E'	0.48	0.86	0.86	0.93	1	1	0.93	1
2MC	0.4	0.77	0.92	1	0.93	0.93	1	0.93
Lig tf	0.48	0.86	0.86	0.93	1	1	0.93	1

Fig 7.35 : Analysis of RAPD bands obtained by OPA 19



As shown in figure 7.35, the fragment profile generated from DNA sample of all 8 isolates with primer OPA 19 gave 22 bands, out of which 17 bands were polymorphic. This primer gave 77% polymorphism. The RAPD bands ranged from 72-1594 base pairs. Out of total 8 RAPD phenotypes, 6 phenotypes were different, giving the primer 75% differentiating power. As seen in fig. 7.36, three isolates – Lig Tf, HGM 17 and E' gave 100% similar phenotype, while SRD 5 was different from all other isolates. With OPA 19, 44% to 100% similar profiles were obtained (table 7.19).

Fig 7.36 : Dendrogram of isolates as obtained by OPA 19

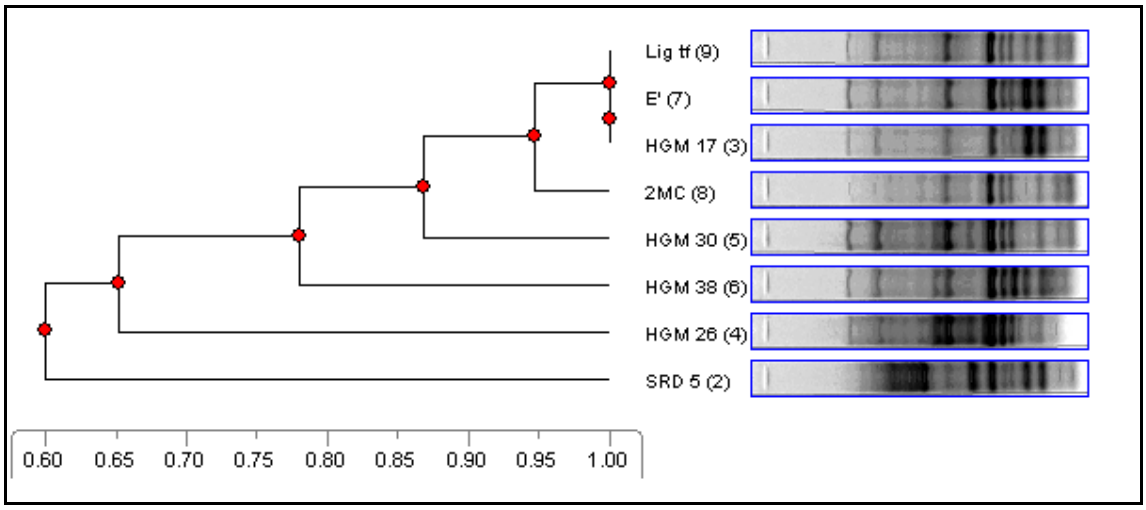
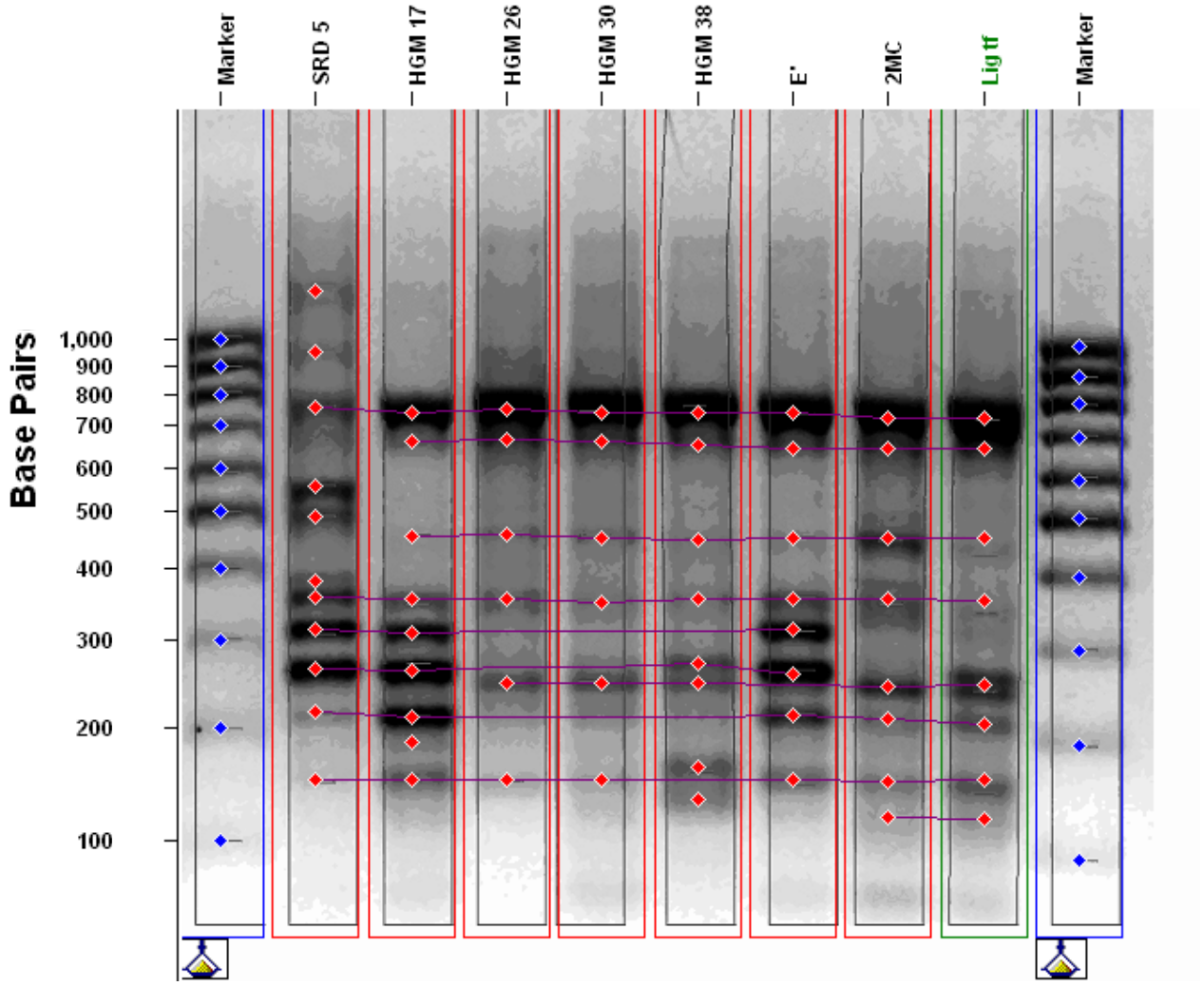


Table 7.19: Similarity matrix given by OPA 19

Lane	SRD 5	HGM 17	HGM 26	HGM 30	HGM 38	E'	2MC	Lig tf
SRD 5	1	0.67	0.44	0.64	0.5	0.67	0.61	0.67
HGM 17	0.67	1	0.61	0.86	0.8	1	0.95	1
HGM 26	0.44	0.61	1	0.75	0.7	0.61	0.64	0.61
HGM 30	0.64	0.86	0.75	1	0.76	0.86	0.9	0.86
HGM 38	0.5	0.8	0.7	0.76	1	0.8	0.74	0.8
E'	0.67	1	0.61	0.86	0.8	1	0.95	1
2MC	0.61	0.95	0.64	0.9	0.74	0.95	1	0.95
Lig tf	0.67	1	0.61	0.86	0.8	1	0.95	1

Fig 7.37 : Analysis of RAPD bands obtained by OPA 20



As shown in figure 7.37, the fragment profile generated from DNA sample of all 8 isolates with primer OPA 20 gave 18 bands, out of which 16 bands were polymorphic. The RAPD bands ranged from 133-1169 base pairs. Out of total 8 RAPD phenotypes, 6 phenotypes were different, giving the primer 75% differentiating power. As seen in fig. 7.38, isolates – Lig Tf and 2MC have shown 100% similar phenotype and HGM 26 and HGM 30 have also shown 100% phenotype. While SRD 5 was different from all other isolates. With OPA 20, 32% to 100% similar profiles were obtained (table 7.20).

Fig 7.38 : Dendrogram of isolates as obtained by OPA 20

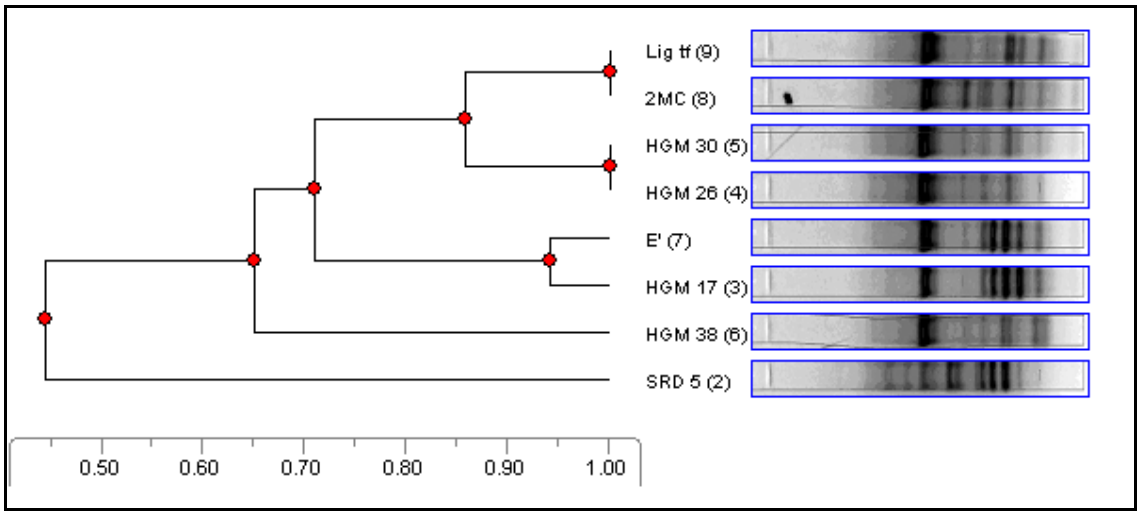


Table 7.20: Similarity matrix given by OPA 20

Lane	SRD 5	HGM 17	HGM 26	HGM 30	HGM 38	E'	2MC	Lig tf
SRD 5	1	0.6	0.35	0.35	0.32	0.63	0.42	0.42
HGM 17	0.6	1	0.67	0.67	0.59	0.94	0.71	0.71
HGM 26	0.35	0.67	1	1	0.71	0.71	0.86	0.86
HGM 30	0.35	0.67	1	1	0.71	0.71	0.86	0.86
HGM 38	0.32	0.59	0.71	0.71	1	0.63	0.63	0.63
E'	0.63	0.94	0.71	0.71	0.63	1	0.75	0.75
2MC	0.42	0.71	0.86	0.86	0.63	0.75	1	1
Lig tf	0.42	0.71	0.86	0.86	0.63	0.75	1	1

7.3.3 POOLED DATA FROM ALL THE RAPD PROFILES

The PCR amplicon of the 8 isolates produced by 18 RAPD primers gave total 352 bands out of which 307 bands were polymorphic (Table 7.21). The primers generated amplicons in the size range of 23 bp by OPA 5 to 1792 bp by OPA 17. The highest range of band size was also shown by OPA 17, which generated RAPD band ranging from 72 bp to 1792 bp. The minimum 11 bands were produced by primer OPA 2 and maximum 37 bands were generated by OPA 4. The same two primers also gave the lowest (6) and highest number (37) of polymorphic bands respectively. OPA 4, OPA 5 and OPA 14 have given 100% polymorphism, where all the bands obtained were different. However the least polymorphism was given by OPA 2, where 5 out of 11 bands obtained were similar.

When banding pattern (RAPD phenotypes) given by each primer was studied, it was found out that different primers had low or high differentiating ability between the studied strains. Out of the 18 primers used, 9 primers could differentiate all the 8 strains into different phenotypes (Table 7.22). Here OPA 2 gave 67% - 100% similarity coefficient in phenotypes, which is highest among all the primers used (Table 7.23). This indicates that most similar bands among all the 8 isolates were obtained here.

The least similarity in bands was achieved with OPA 6, which gave only 38 to 77% similarity in banding pattern of all the isolates. Moreover OPA 14 is the only primer, which has given similarity coefficient ranging from 0-100%. Here Isolates SRD 5 and HGM 38 show 0% similarity and isolates Lig tf and 2MC show 100% similarity coefficient. Similar result was obtained by Ni *et al.* (2008), who found that RAPD-PCR reveals considerable genomic diversity within *A. ferrooxidans* and its strains have a wide similarity coefficient range from 0% to 98%. Kelly and Wood (2000) have also mentioned that *A. ferrooxidans* strains exhibit 0% to 97% similarity in their genomic patterns.

Table 7.21: Summery of RAPD profiles obtained by 18 primers

Primer	Total no of bands	Total no. of polymorphic bands	Amplified product (Base pairs)	Percent polymorphism
OPA 2	11	6	214-765	54
OPA 3	25	23	93-1122	92
OPA 4	37	37	157-1483	100
OPA 5	22	22	23-877	100
OPA 6	29	25	96-1465	86
OPA 7	23	19	33-1171	83
OPA 8	16	13	35-1013	81
OPA 10	12	11	146-1701	92
OPA 11	17	12	61-1295	71
OPA 12	16	12	110-1150	75
OPA 13	17	15	126-1282	88
OPA 14	16	16	104-1354	100
OPA 15	22	21	62-1377	95
OPA 16	12	9	102-1043	75
OPA 17	21	19	76-1792	90
OPA 18	16	14	136-1375	87
OPA 19	22	17	72-1594	77
OPA 20	18	16	133-1169	89

Table 7.22: Phenotype observed by each primer along with its differentiating power

Primer	Sequence	Total no. of phenotype	No. of RAPD phenotype	Differentiating power (%)
OPA 2	TGC CGA GCT G	8	7	87
OPA 3	AGT CAG CCA C	8	8	100
OPA 4	AAT CGG GCT G	8	8	100
OPA 5	AGG GGT CTT G	8	8	100
OPA 6	GGT CCC TGA C	8	8	100
OPA 7	GAA ACG GGT G	8	7	87
OPA 8	GTG ACG TAG G	8	8	100
OPA 10	GTG ATC GCA G	8	8	100
OPA 11	CAA TCG CCG T	8	6	75
OPA 12	TCG GCG ATA G	8	7	87
OPA 13	CAG CAC CCA C	8	8	100
OPA 14	TCT GTG CTG G	8	7	87
OPA 15	TTC CGA ACC C	8	8	100
OPA 16	AGC CAG CGA A	8	5	62
OPA 17	GAC CGC TTG T	8	8	100
OPA 18	AGG TGA CCG T	8	5	62
OPA 19	CAA ACG TCG G	8	6	75
OPA 20	GTT GCG ATC C	8	6	75

Table 7.23: Range of similarity among isolates

Primer	Lowest similarity obtained	Highest similarity obtained	Most similar isolates	Least similar isolates
OPA 2	67	100	HGM 26-HGM 38	2MC-E'
OPA 3	19	94	HGM 26-HGM 30	HGM 17
OPA 4	0	97	HGM 17-HGM 38, HGM 26-SRD 5	HGM 30
OPA 5	0	91	HGM 38-SRD 5	HGM 26
OPA 6	38	77	HGM 17-E', HGM 26 – HGM 38	SRD 5
OPA 7	48	100	HGM 26-HGM 30	SRD 5
OPA 8	38	93	HGM 17-SRD 5	HGM 26
OPA 10	17	92	HGM 26-HGM 38	SRD 5
OPA 11	52	100	Lig Tf'-HGM 17-E'	SRD 5
OPA 12	40	100	Lig Tf'-HGM 30	SRD 5
OPA 13	44	95	HGM 26-HGM 30	SRD 5
OPA 14	0	100	Lig Tf'-2MC	SRD 5, HGM 17
OPA 15	24	92	HGM 17-E'	HGM 26
OPA 16	40	100	Lig Tf'-HGM 30-E'-2MC	SRD 5
OPA 17	33	95	Lig Tf-2MC	SRD 5
OPA 18	32	100	Lig Tf'-HGM 38-E', HGM 30-2MC	SRD 5
OPA 19	44	100	Lig Tf'-HGM 17-E'	SRD 5
OPA 20	32	100	Lig Tf-2MC, HGM 26-HGM 30	SRD 5

On the basis of RAPD patterns obtained, Jaccard coefficient was obtained. This indicates the highest similarity coefficient 0.722 between HGM 17 and E' isolates (Table 7.24). The next highest similarity, was between Lig Tf and E' which was 0.713. Apart from these, higher similarity was also observed between HGM 38 – E', Lig Tf – HGM 17 and Lig-Tf – 2MC. The least similar isolate among all was SRD 5.

Table 7.24 : Proximity matrix of all isolates by Jaccard coefficient

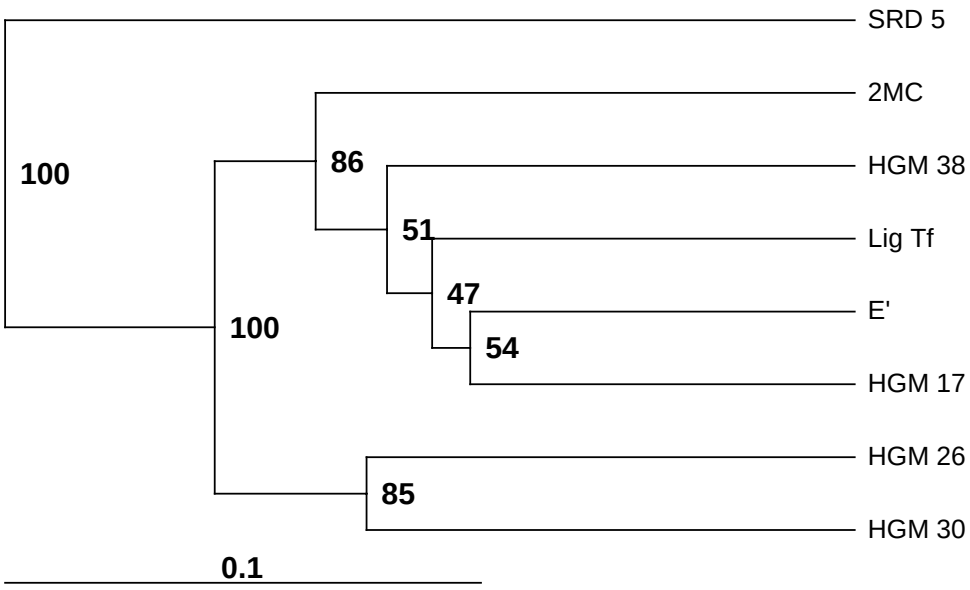
	Jaccard Measure							
	E	HGM17	HGM26	HGM30	HGM38	LIG_TF	MC2	SRD5
E		0.722	0.505	0.579	0.674	0.713	0.617	0.529
HGM17	0.722		0.523	0.556	0.674	0.684	0.601	0.554
HGM26	0.505	0.523		0.660	0.608	0.589	0.532	0.409
HGM30	0.579	0.556	0.660		0.635	0.624	0.623	0.436
HGM38	0.674	0.674	0.608	0.635		0.667	0.614	0.471
LIG_TF	0.713	0.684	0.589	0.624	0.667		0.693	0.477
MC2	0.617	0.601	0.532	0.623	0.614	0.693		0.450
SRD5	0.529	0.554	0.409	0.436	0.471	0.477	0.450	

When all the bands obtained by each primer were put together to compare the similarity and differences between the isolates, dendrograms constructed from pooled data was obtained as shown in figure 7.39. It indicates that isolates E' and HGM 17 are most similar among all isolates tested. Inspite of these two isolates belong to two different geographical location and different mining environments. Isolate E' is obtained from polymetallic mining environment where copper, zinc and lead are available in considerable amount. While HGM 17, has been isolated from arsenopyrite leaching environments. As against this isolate SRD 5 which has been isolated from Amabamata polymetallic mine was found to be most dissimilar of all. All other isolates showed difference in phenotype from each other.

Karavaiko *et al.* (2003) failed to reveal geographical correlation between the diversity of *A. ferrooxidans* strains and their origin on the basis of restriction pattern of chromosomal DNA or phylogenetic analysis of 16S rRNA genes or DNA-DNA hybridization. But they indicated the existence of a certain degree of correlation between the similarity of the genomes of *A. ferrooxidans* strains and the mineralogical characteristics of their habitat and therefore each indigenous *A. ferrooxidans* strain isolated from an econiche with a particular mineralogical composition of oxidizable substrates was characterized by its own chromosomal DNA restriction pattern. Similarly, Ni *et al.* (2008) found no clear correlation between the genotypic group of the *Acidithiobacillus* sp. strains and either the geographical location or type of habitat/sample from which the strains were isolated.

However scientists have reported that adaptation to different substrate/energy source on metal ions exerts change in gene sequence and plasmid number. Kondrat'eva *et al.* (2002) reported that change in plasmid profiles was observed in some *A. ferrooxidans* strains (TFZ, TFI-Fe, TFV-1-Cu) with experimentally enhanced resistance to Zn^{2+}, Fe^{3+} and Cu^{2+} respectively, as compared to initial strains.

Fig. 7.39: Dendrogram illustrating the clustering of *A. ferrooxidans* isolates based on their RAPD profiles which were evaluated by using Nei and Li's co-efficient and the Unweighted pair group method of arithmetic means (UPGMA) algorithm



Similarly Novo *et al.* (2000) reported changes in total protein synthesis patterns by polyacrylamide gel electrophoresis for *T. ferrooxidans* LR cells grown in the absence and presence of different heavy metals like 200 mM copper, 600 mM cadmium, 600 mM nickel and 600 mM zinc.

To evaluate the diversity among strains of *A. ferrooxidans* isolates, the comparison of our environmental isolates and known species is required. By using physiological growth pattern and colony characteristics, we were able to achieve generic identification of the isolate. Reliable identification of genera and species required the application of DNA sequence analysis.

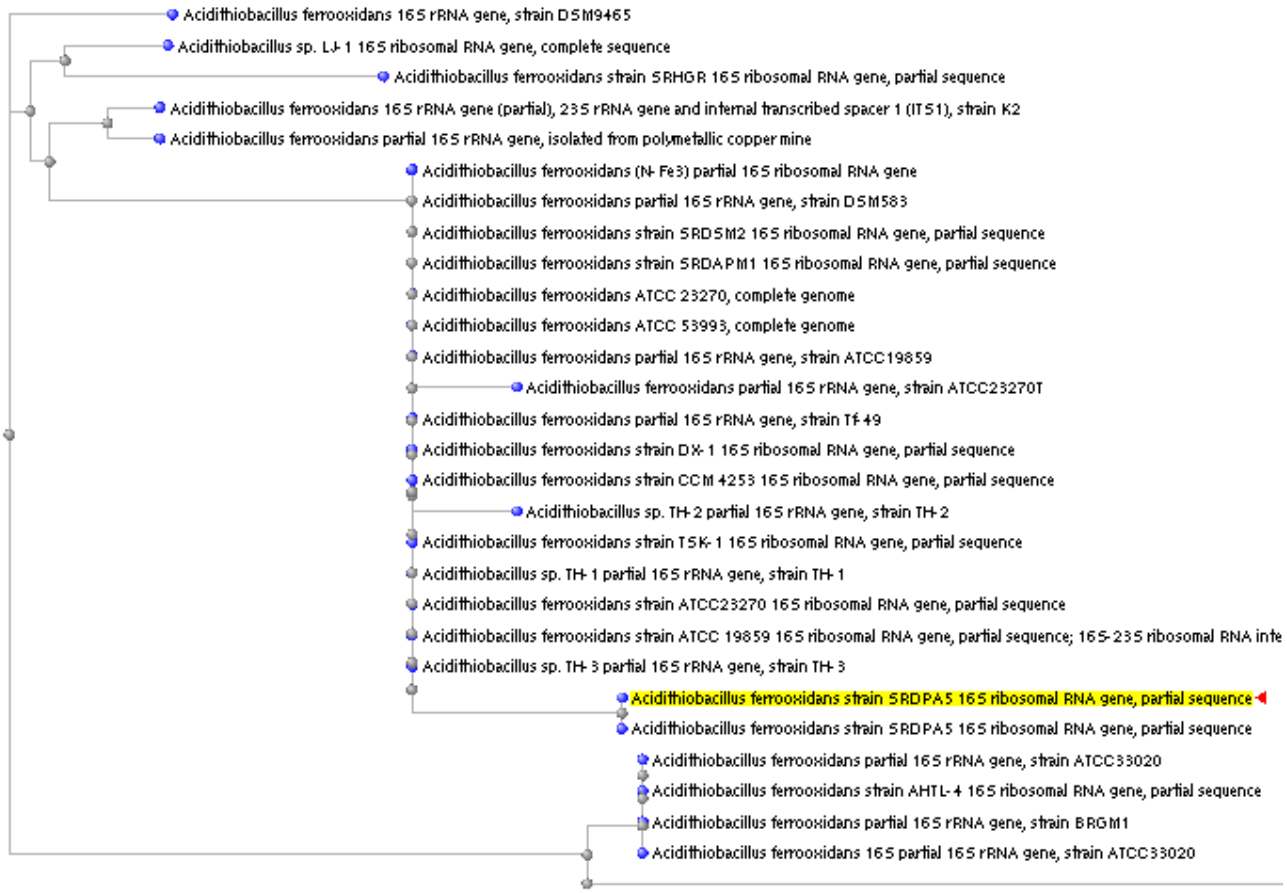
7.3.4 16S rRNA GENE SEQUENCING

In this study, we used 16S rRNA gene sequence phylogenetic analysis to study the strain diversity of *A. ferrooxidans*. It is known that microbes with 16S rRNA gene sequence similarity upto 97% identical should be considered as members of the same genus.

7.3.4.1 PHYLOGENETIC ANALYSIS OF ISOLATE SRD 5 (SRDPA5)

Analysis of 960 bp long partial nucleotide sequence of SRD5-16S rRNA gene was done to find identification as well as similarity with other bacterial strains in the database. This comparison with Genbank, EMBL, DDBJ and RDP databases yielded a group of *Acidithiobacillus ferrooxidans* strains, whose 16S rRNA gene nucleotide sequence matched as high as 98%. The isolate was designated as SRDPA5 and phylogenetic tree showing this isolate with related strains is shown in figure 7.40. The phylogenetic tree analysis with the strains belonging to γ-proteobacteria showed that the strain SRD 5 shares 99% similarity with *Acidithiobacillus ferrooxidans* strain A2 (Accession number FN686784.1). The 16S rRNA gene sequence is submitted to the NCBI database under accession number **HM116944.1.**

Figure 7.40: Phylogenetic tree from 16S rRNA gene sequence of SRD 5.



Acidithiobacillus ferrooxidans strain SRDPA5 16S ribosomal RNA gene, partial sequence

GenBank: HM116944.1

FASTA Graphics

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ORGANISM    Acidithiobacillus ferrooxidans
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            Acidithiobacillales; Acidithiobacillaceae; Acidithiobacillus.
REFERENCE   1  (bases 1 to 960)
AUTHORS     Dave,S.R. and Ansari,P.A.
TITLE       Direct Submission
JOURNAL     Submitted (12-APR-2010) Department of Microbiology, Gujarat
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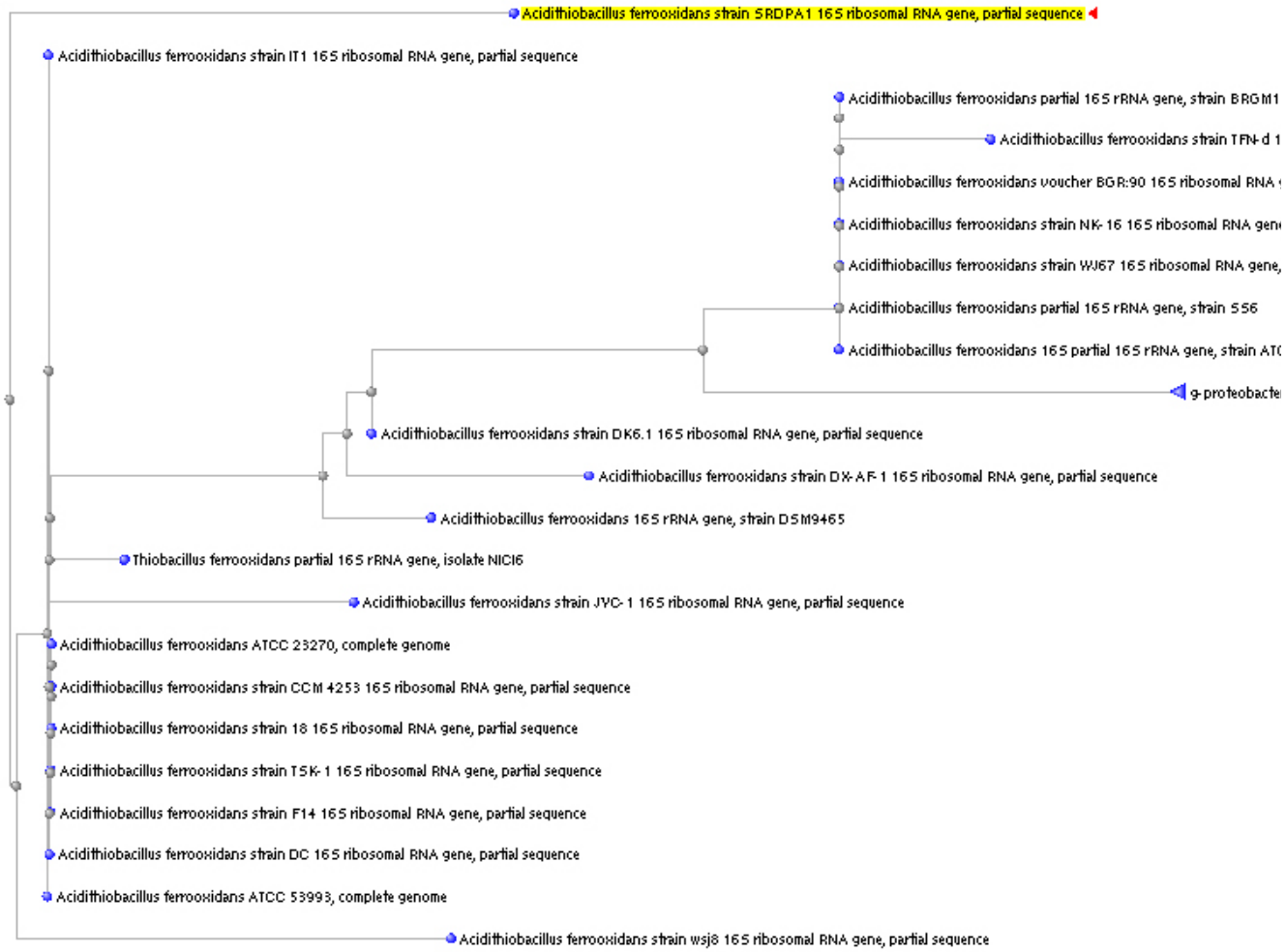
7.3.4.2 PHYLOGENETIC ANALYSIS OF ISOLATE 2MC (SRDPA1)

Partial nucleotide sequence of 16S rRNA gene of isolate 2MC was 857 bp long, which was analyzed by nucleotide blast in NCBI website (www.ncbi.nlm.nih.gov) and its identity was matched with other bacterial strains in the database. This comparison with Genbank, EMBL, DDBJ and RDP databases yielded a group of *Acidithiobacillus ferrooxidans* strains, out of which maximum similarity (98%) was obtained with *Acidithiobacillus ferrooxidans* partial 16S rRNA gene, strain IT1 (Accession number IN224813.1). The 16S rRNA gene sequence is submitted to the NCBI database under accession number **HQ262544.1** and designated as strain SRDPA1. Phylogenetic tree showing this isolate with related strains is shown in figure 7.41.

Figure 7.41: Phylogenetic tree from 16S rRNA gene sequence of 2MC



Acidithiobacillus ferrooxidans strain SRDPA1 16S ribosomal RNA gene, partial sequence

GenBank: HQ262544.1

FASTA Graphics

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            RNA gene, partial sequence.
ACCESSION   HQ262544
VERSION     HQ262544.1  GI:309753462
SOURCE      Acidithiobacillus ferrooxidans
ORGANISM    Acidithiobacillus ferrooxidans
            Bacteria; Proteobacteria; Gammaproteobacteria;
            Acidithiobacillales; Acidithiobacillaceae; Acidithiobacillus.
REFERENCE   1  (bases 1 to 857)
AUTHORS     Dave,S.R. and Ansari,P.A.
TITLE       Acidithiobacillus ferrooxidans isolated from Malanjkhand
            Copper Mine
JOURNAL     Unpublished
REFERENCE   2  (bases 1 to 857)
AUTHORS     Dave,S.R. and Ansari,P.A.
TITLE       Direct Submission
JOURNAL     Submitted (14-SEP-2010) Department of Microbiology, Gujarat
            University, School of Sciences, Navrangpura, Ahmedabad,
            Gujarat 380009, India
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                     /mol_type="genomic DNA"
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                     /isolation_source="water sample"
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                     /country="India: Malanjkhand Copper Mine"
                      rRNA            <1..>857
                     /product="16S ribosomal RNA"
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121 tcttcggacc tcgcgctaag agaggagcct acgtccgatt agctagttgg cggggtaaag
181 gcccaccaag gcgacgatcg gtagctggtc tgagaggacg accagccaca ctgggactga
241 gacacggccc agactcctac gggaggcagc agtggggaat ttttcgcaat gggggcaacc
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361 ggacgaaaag gtgggttcta atacaatctg ctattgacgt gaatccaaga agaagcaccg
421 gctaactccg tgccagcagc cgcggtaata cgggggggtgc aagcgttaat cggaatcact
481 gggcgtaaag ggtgcgtagg cggtacgtta ggtctgtcgt gaaatccccg ggctcaacct
541 gggaatggcg gtggaaaccg gtgtactaga gtatgggaga gggtggtgga attccaggtg
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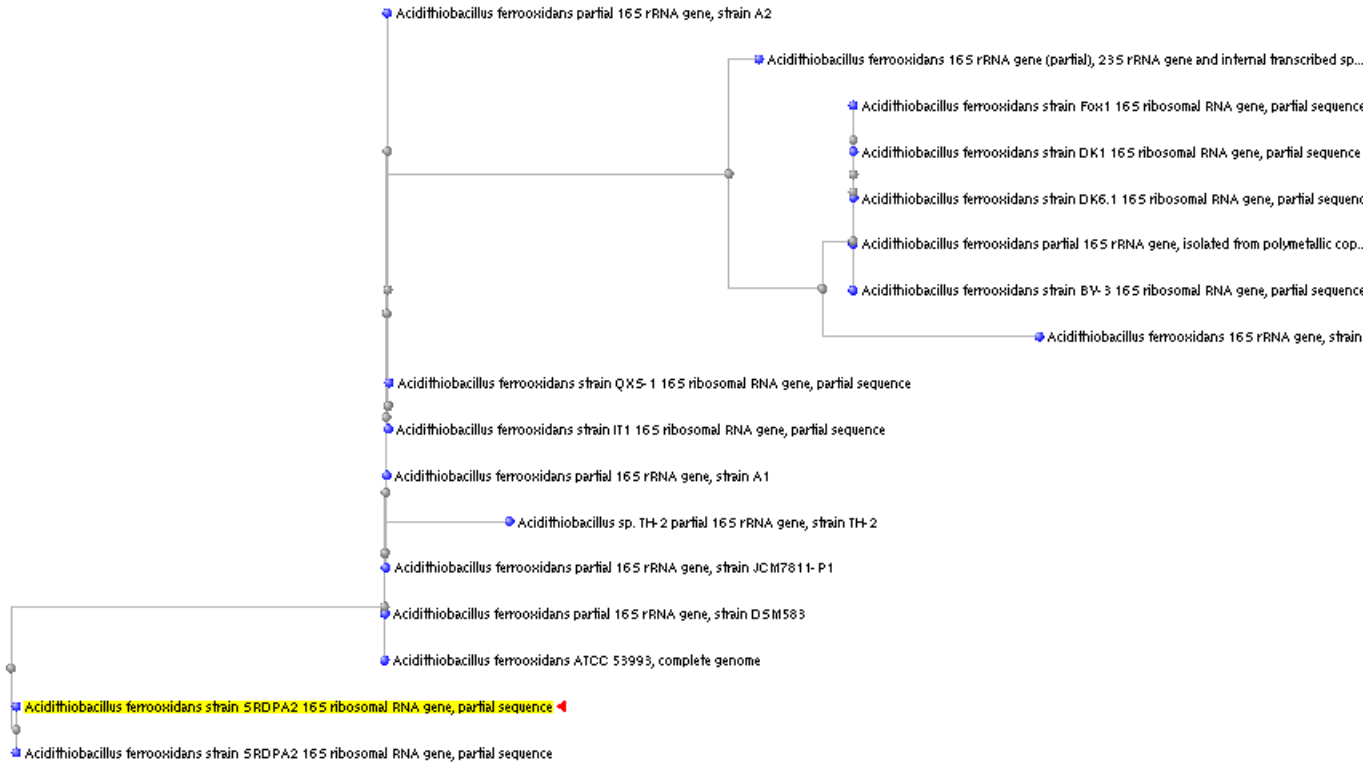
661 cccaaatact gacgctgagg cacgaaagcg tggggagcaa acaggattag ataccctggt
721 agtccacgcc ctaaacgatg aatactagat gtttggtgcc tagcgtactg agtgtcgtag
781 ctacgcgatt aagtattccg cctggaaagt acggacgcaa gttaaaactc aagggaattg

841 acggggccgc acaagcg
```

7.3.4.3 PHYLOGENETIC ANALYSIS OF ISOLATE HGM 30 (SRDPA2)

711 bp long partial nucleotide sequence of 16S rRNA gene of HGM 30 was analyzed to find identification as well as similarity with other bacterial strains in the database. This comparison with Genbank, EMBL, DDBJ and RDP databases yielded a group of closely associated (98% similar) *Acidithiobacillus ferrooxidans* strains. The 16S rRNA gene sequence was submitted to the NCBI database under accession number **HQ262545.1** and designated as strain SRDPA2. Phylogenetic tree showing this isolate with related strains is shown in figure 7.42. The phylogenetic tree analysis with the strains belonging to γ-proteobacteria showed that the strain HGM 30 shares 96% similarity with *Acidithiobacillus ferrooxidans* partial 16S rRNA gene, strain TH-3 (Accession number AM176775.1).

Figure 7.42: Phylogenetic tree from 16S rRNA gene sequence of HGM 30.



Acidithiobacillus ferrooxidans strain SRDPA2 16S ribosomal RNA gene, partial sequence

GenBank: HQ262545.1

FASTA Graphics

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DEFINITION  Acidithiobacillus ferrooxidans strain SRDPA2 16S ribosomal
            RNA gene, partial sequence.
ACCESSION   HQ262545
VERSION     HQ262545.1  GI:309753463
SOURCE      Acidithiobacillus ferrooxidans
ORGANISM    Acidithiobacillus ferrooxidans; Bacteria; Proteobacteria;
            Gammaproteobacteria; Acidithiobacillales;
            Acidithiobacillaceae; Acidithiobacillus.
REFERENCE   1  (bases 1 to 711)
AUTHORS     Dave,S.R. and Ansari,P.A.
TITLE       Acidithiobacillus ferrooxidans isolated from Hutti Gold Mine,
            Karnataka, India
JOURNAL     Unpublished
REFERENCE   2  (bases 1 to 711)
AUTHORS     Dave,S.R. and Ansari,P.A.
TITLE       Direct Submission
JOURNAL     Submitted (14-SEP-2010) Department of Microbiology, Gujarat
            University, School of Sciences, Navrangpura, Ahmedabad,
            Gujarat 380009, India
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181 ccacactggg actgagacac ggcccagact cctacgggag gcagcagtgg ggaatttttc
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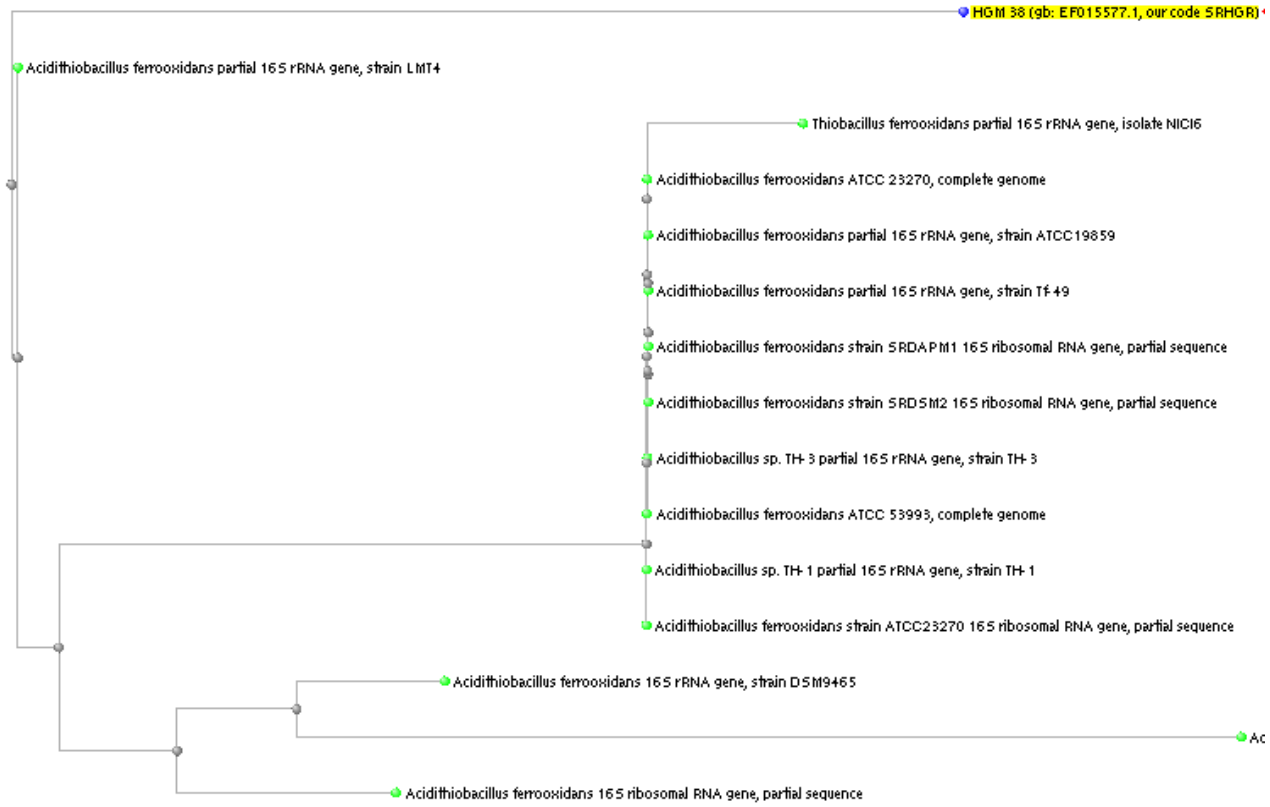
361 caagaagaag caccggctaa ctccgtgcca gcagccgcgg taatacgggg ggtgcaagcg
421 ttaatcggaa tcactgggcg taaagggtgc gtaggcggta cgttaggtct gtcgtgaaat
481 ccccgggctc aacctgggaa tggcggtgga aaccggtgta ctagagtatt ggggagaggg
541 tggtggaatt ccaggtgtta gcgggtgaaa atgcgtagag atctgggagg aaacatcagt
601 tggcgaaggg cggccaccct gggccccaat actgacgctg aggcacgaaa ggcgtggggg
661 agccaaaacc aggaattaga tacccttggt tagtccaacg ccctaaaacg a
```

7.3.4.4 PHYLOGENETIC ANALYSIS OF ISOLATE HGM 38 (SRHGR)

1344 bp long partial nucleotide sequence of 16S rRNA gene of HGM 38 was analyzed to find identification as well as similarity with other bacterial strains in the database. The 16S rRNA gene sequence was submitted to the NCBI database by Dr. Dave and Dr. Tipre, under accession number **EF015577.1** and designated as strain SRHGR. Its comparison with Genbank, EMBL, DDBJ and RDP databases yielded 98% match with 16SrRNA gene nucleotide sequence of *Acidithiobacillus ferrooxidans* ATCC 53993, (accession number CP001132.1) and *Acidithiobacillus ferrooxidans* ATCC 23270, complete genome (accession number CP001219.1). However its closest match was found with *Acidithiobacillus ferrooxidans* strain LMT4 (accession number AM502931.1). Phylogenetic tree showing this isolate with related strains is shown in figure 7.43.

Figure 7.43: Phylogenetic tree of 16S rRNA gene sequence of HGM 38.



Acidithiobacillus ferrooxidans strain SRHGR 16S ribosomal RNA gene, partial sequence

GenBank: EF015577.1

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DEFINITION  Acidithiobacillus ferrooxidans strain SRHGR 16S ribosomal
            RNA gene,partial sequence.
ACCESSION   EF015577
VERSION     EF015577.1  GI:116272522
SOURCE      Acidithiobacillus ferrooxidans
ORGANISM    Acidithiobacillus ferrooxidans
            Bacteria; Proteobacteria; Gammaproteobacteria;
            Acidithiobacillales; Acidithiobacillaceae; Acidithiobacillus
REFERENCE   1  (bases 1 to 1344)
AUTHORS     Dave,S.R. and Tipre,D.R.
TITLE       Bioleaching Bacteria
JOURNAL     Unpublished
REFERENCE   2  (bases 1 to 1344)
AUTHORS     Dave,S.R. and Tipre,D.R.
TITLE       Direct Submission
JOURNAL     Submitted (20-SEP-2006) Department of Microbiology, Gujarat
            University, School of Sciences, Gujarat University,
            Ahmedabad, Gujarat 380009, India
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                     /db_xref="taxon:920"
                     /country="India"
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                      rRNA            <1..>1344
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      121 ctaataccgc atgagccctg agggggaaag cgggggatct tcggacctcg cgctaagaga
      181 ggagcctacg tccgattagc tagttggcgg ggtaaaggcc caccaaggcg acgatcggta
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      541 tacgttaggt ctgtcgtgaa atccccgggc tcaacctggg aatggcggtg gaaaccggcg
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     1021 gtgtcgtgag atgttgggtt aagtcccgca acgagcgcaa cccttgtcct tatttgccag
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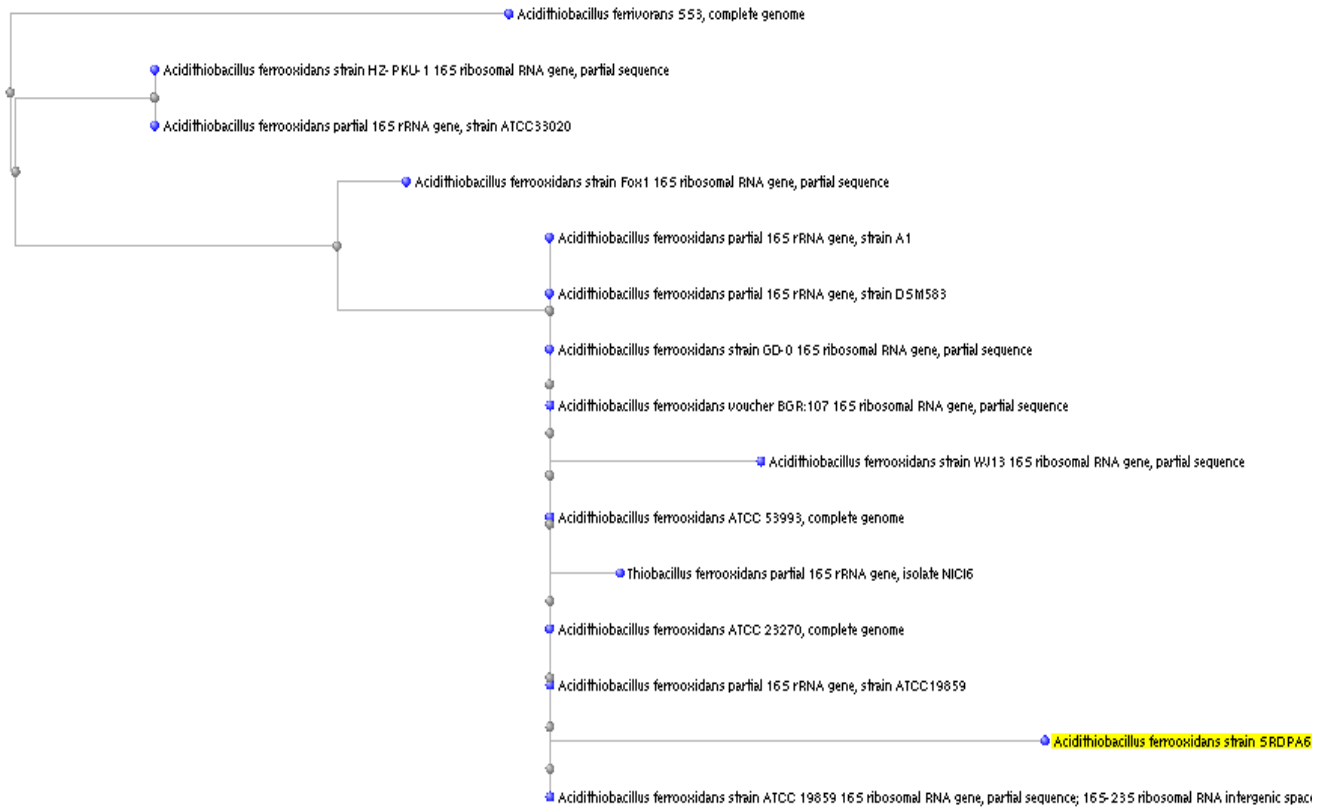
     1321 cgttcccggg ccttgtacac acat
```

7.3.4.5 PHYLOGENETIC ANALYSIS OF ISOLATE E' (SRDPA6)

16S rRNA gene partial nucleotide sequence of isolate E' was 680 bp long. It was analyzed to find identification as well as similarity with other bacterial strains in the database. This comparison with Genbank, EMBL, DDBJ and RDP databases yielded 99% similarity with 16S rRNA gene sequence of *Acidithiobacillus ferrooxidans* ATCC 53993, (accession number CP001132.1) and *Acidithiobacillus ferrooxidans* ATCC 23270, complete genome (accession number CP001219.1) The 16S rRNA gene sequence was submitted to the NCBI database under accession number **JF701445.1** and designated as strain SRDPA6. Phylogenetic tree showing this isolate with related strains is shown in figure 7.44. The phylogenetic tree analysis with the strains belonging to γ-proteobacteria showed that the strain E' shared 99% similarity with *Acidithiobacillus ferrooxidans* strain A1 (Accession number FN686783.1).

Figure 7.44: Phylogenetic tree of 16S rRNA gene sequence of isolate E'.



Acidithiobacillus ferrooxidans strain SRDPA6 16S ribosomal RNA gene, partial sequence

GenBank: JF701445.1

FASTA Graphics

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DEFINITION  Acidithiobacillus ferrooxidans strain SRDPA6 16S ribosomal
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ACCESSION   JF701445
VERSION     JF701445.1  GI:346230948
KEYWORDS    .
SOURCE      Acidithiobacillus ferrooxidans
ORGANISM    Acidithiobacillus ferrooxidans
            Bacteria; Proteobacteria; Gammaproteobacteria;
            Acidithiobacillales; Acidithiobacillaceae; Acidithiobacillus.
REFERENCE   1  (bases 1 to 680)
  AUTHORS   Dave,S.R. and Ansari,P.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (16-MAR-2011) Department of Microbiology, Gujarat
            University, School of Sciences, Navrangpura, Ahmedabad,
            Gujarat 380009, India
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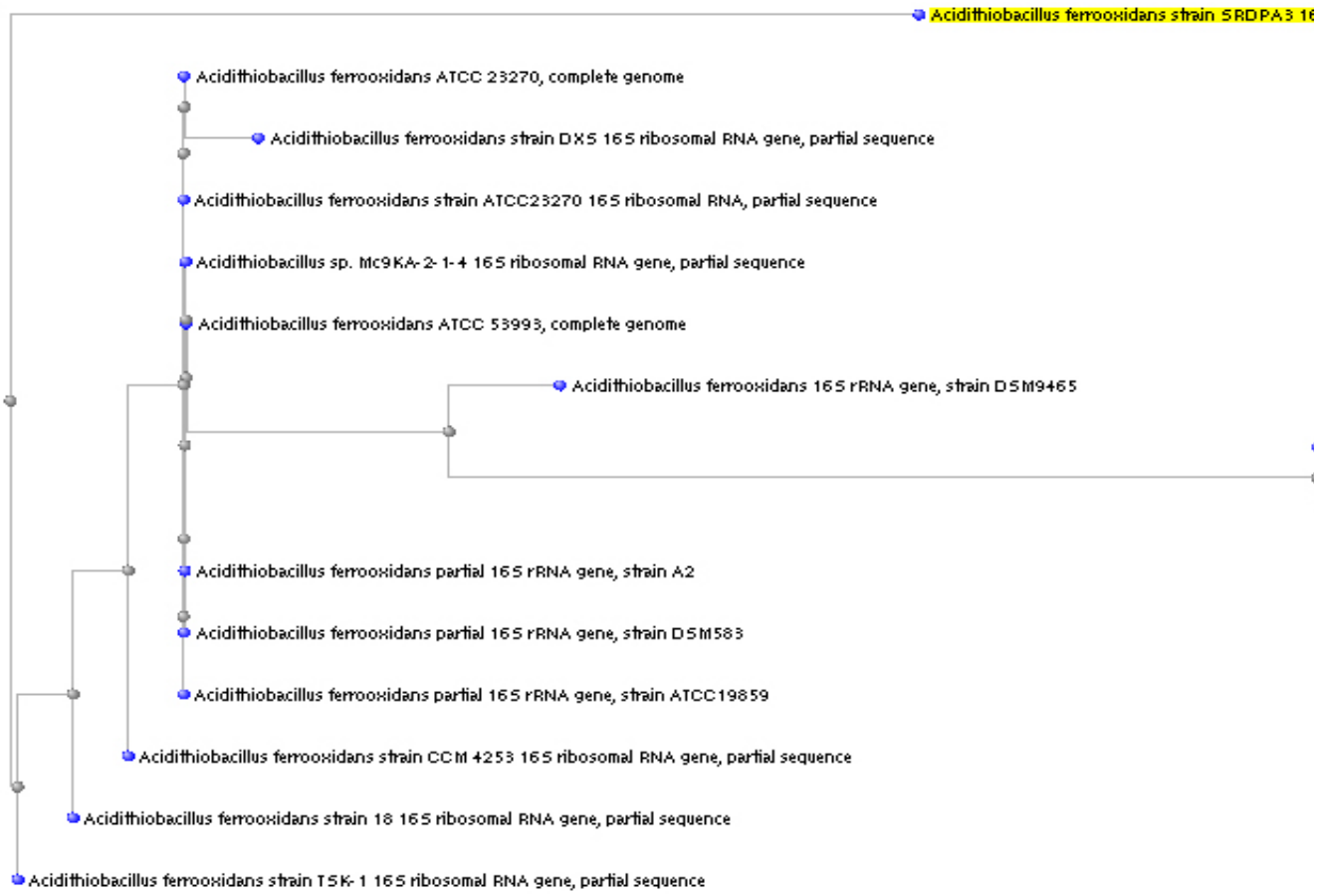
661 gagcaaacag gagtagatgc
```

http://www.ncbi.nlm.nih.gov/nuccore/JF701445.1

7.3.4.6 PHYLOGENETIC ANALYSIS OF ISOLATE HGM 17 (SRDPA3)

904 bp long partial nucleotide sequence of 16S rRNA gene of HGM 17 was blast searched in NCBI website (www.ncbi.nlm.nih.gov) and its identity was matched with other bacterial strains available in the database. This comparison with Genbank, EMBL, DDBJ and RDP databases yielded 97% similarity with *Acidithiobacillus ferrooxidans* ATCC 23270 (CP001219.1), *A. ferrooxidans* ATCC 53993 (CP001132.1) and *A. ferrooxidans* partial 16S rRNA gene, strain ATCC19859 (AJ457808.3). The 16S rRNA gene sequence was submitted to the NCBI database under accession number **JF701443.1** and designated as strain SRDPA3. Phylogenetic tree showing this isolate with related strains is given in figure 7.45. Isolate HGM 17 showed highest similarity (98%) with partial 16S rRNA gene of *Acidithiobacillus ferrooxidans* TSK-1 strain (Accession number EF210567.1).

Figure 7.45: Phylogenetic tree from 16S rRNA gene sequence of HGM 17.



Acidithiobacillus ferrooxidans strain SRDPA3 16S ribosomal RNA gene, partial sequence

GenBank: JF701443.1

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DEFINITION  Acidithiobacillus ferrooxidans strain SRDPA3 16S ribosomal
RNA gene, partial sequence.
ACCESSION   JF701443
VERSION     JF701443.1  GI:346230946
SOURCE      Acidithiobacillus ferrooxidans
ORGANISM    Acidithiobacillus ferrooxidans
            Bacteria; Proteobacteria; Gammaproteobacteria;
            Acidithiobacillales; Acidithiobacillaceae; Acidithiobacillus
REFERENCE   1  (bases 1 to 904)
  AUTHORS   Dave,S.R., Tipre,D.R. and Ansari,P.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (16-MAR-2011) Department of Microbiology, Gujarat
            University, School of Sciences, Navrangpura, Ahmedabad,
            Gujarat, 380009, India
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901 ttac
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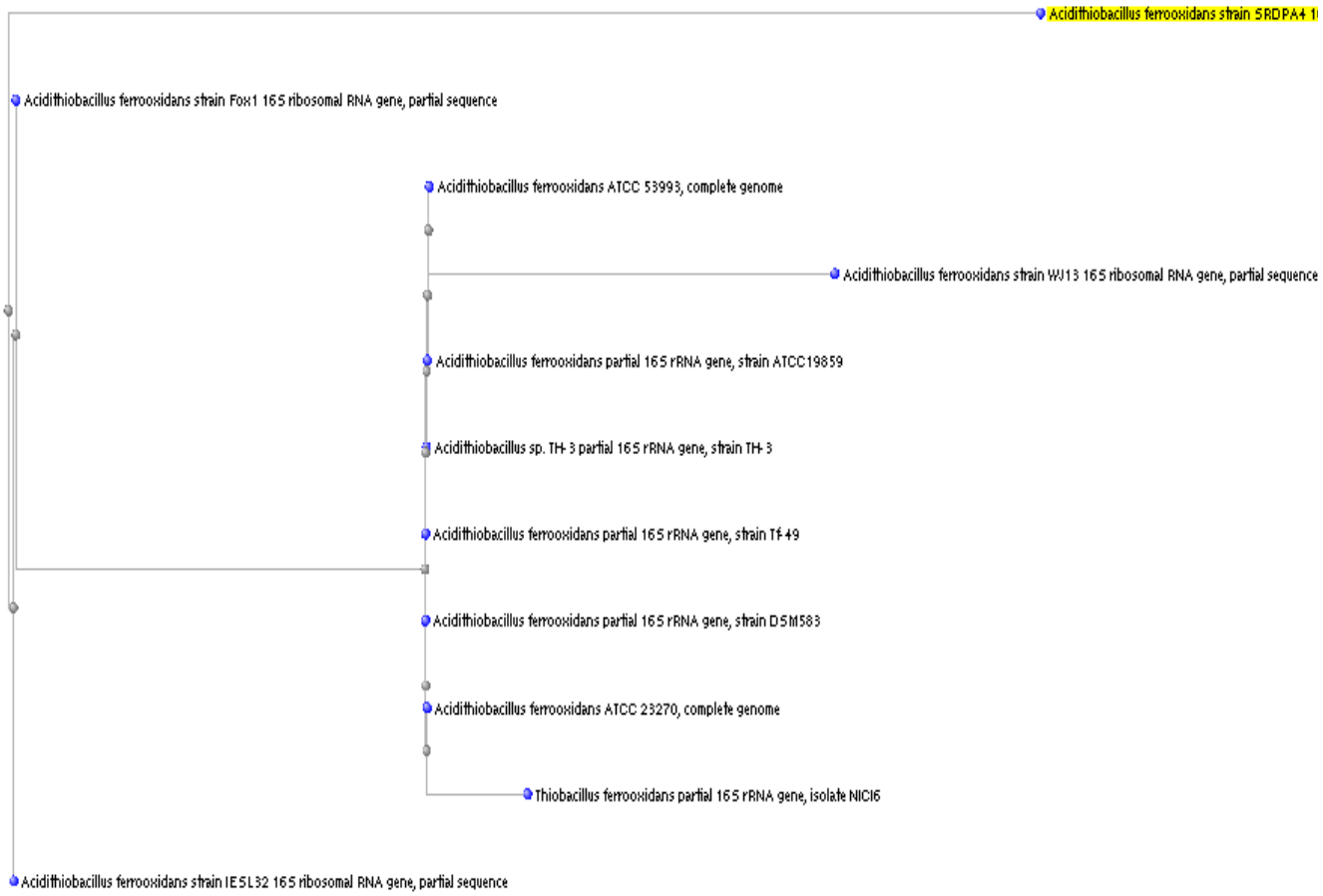
http://www.ncbi.nlm.nih.gov/nuccore/JF701443.1

7.3.4.7 PHYLOGENETIC ANALYSIS OF ISOLATE HGM 26 (SRDPA4)

952 bp long partial nucleotide sequence of 16S rRNA gene of HGM 26 was blast searched in NCBI website (www.ncbi.nlm.nih.gov) and its identity was matched with other bacterial strains available in the database. This comparison with Genbank, EMBL, DDBJ and RDP databases yielded 98% similarity with *Acidithiobacillus ferrooxidans* IESL32 strain (Accession number HQ902071.1). The 16S rRNA gene sequence was submitted to the NCBI database under accession number **JF701444.1** and designated as strain SRDPA4. Phylogenetic tree showing this isolate with related strains is given in figure 7.46.

Figure 7.46: Phylogenetic tree from 16S rRNA sequence of HGM 26.



Acidithiobacillus ferrooxidans strain SRDPA4 16S ribosomal RNA gene, partial sequence

GenBank: JF701444.1

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DEFINITION  Acidithiobacillus ferrooxidans strain SRDPA4 16S ribosomal
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ACCESSION   JF701444
VERSION     JF701444.1  GI:346230947
SOURCE      Acidithiobacillus ferrooxidans
ORGANISM    Acidithiobacillus ferrooxidans
            Bacteria; Proteobacteria; Gammaproteobacteria;
            Acidithiobacillales; Acidithiobacillaceae; Acidithiobacillus
REFERENCE   1  (bases 1 to 952)
  AUTHORS   Dave,S.R., Tipre,D.R. and Ansari,P.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (16-MAR-2011) Department of Microbiology, Gujarat
            University, School of Sciences, Navrangpura, Ahmedabad,
            Gujarat, 380009, India
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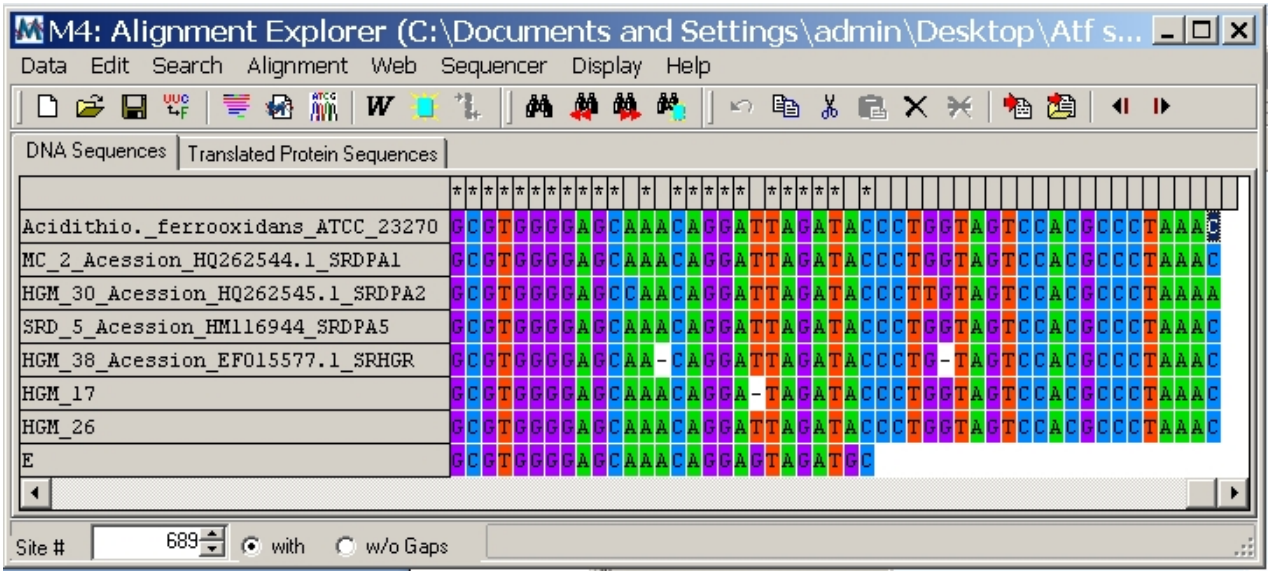
421 gtgaatccaa gaagaagcac cggctaactc cgtgccagca gccgcggtaa tacgggggt
481 gcaagcgtta atcggaatca ctgggcgtaa agggtgcgta ggcggtacgt taggtctgtc
541 gtgaaatccc cgggctcaac ctgggaatgg cggtggaaac cggcgcacta gagtatggga
601 gagggtggtg gaattccagg tgtagcggtg aaatgcgtag agatctggag gaacatcagt
661 ggcgaaggcg gccacctggc ccaatactga cgctgaggca cgaaagcgtg gggagcaaac
721 aggattagat accctggtag tccacgccct aaacgatgaa tactatatgt ttggtgccta
781 gcgtactgag tgtcgnagct cacgcgataa gtattccgcc tgggaagtac ggccgcaggt
841 taaaactcaa ggaattgacn ggggcccgca caagcggtgg agcagtgttt attcgatgca
901 acgcgaagaa ccttacctgg cttgacatgt ccggaattct gcagagatgc gg
```

http://www.ncbi.nlm.nih.gov/nuccore/JF701444.1

Figure 7.47: Alignment of 16S rRNA gene nucleotide sequences of our *A. ferrooxidans* isolates with its Type strain ATCC 23270.

All our sequences showed 97% or more similarity to *A. ferrooxidans* type strain ATCC 23270 (gb accession no. CP001219). Isolate 2_MC and SRD 5 were 100% similar to it, while HGM 17, HGM 26 and HGM 38 were 99.85%, 99.41% and 99.26% similar respectively. Isolate HGM 30 was most dissimilar of all and it showed 98.37% similarity (Data not shown).



7.3.5 PHYLOGENETIC TREE CONSTRUCTED FROM 16S rRNA GENE SEQUENCES

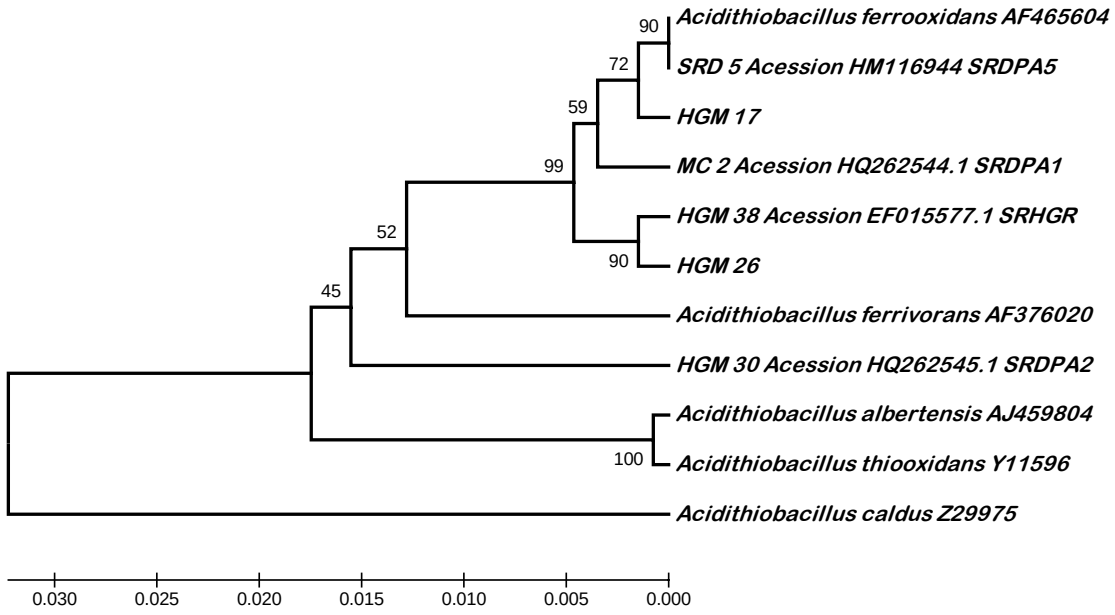
Comparative analysis of PCR amplified 16S rRNA gene sequences of different *Acidithiobacillus* isolates was done. The sequences were initially analyzed using the BLAST search facility (www.ncb.nlm.nih.gov/blast/blast.cgi). Identity of the organisms was confirmed with the standard 16S rRNA gene sequence of *Acidithiobacillus ferrooxidans* Type strain ATCC 23270 available at EzTaxon server 2.1(147.47.212.35/8080). All our strains were identical with Type strain ATCC 23270. Sequences of our organisms and reference strain were edited to get uniformity in experiment and hence a final sequence length of 689 bp was achieved and phylogentic tree was constructed using MEGA 3.1, which is shown in fig. 7.48. Phylogenetic tree showed the relationship between the 16S rRNA gene sequences of this taxonomical group. (Bacteria; Proteobacteria; Gamma proteobacteria; Acidithiobacillales; Acidithiobacillaceae;). The sequence data of other *Acidithiobacillus* type species were recovered from genebank and included in the tree. Phylogenic tree reconstruction analysis was done by UPGMA method. The bootstrap values from 1000 replications are shown at each of the branch points on the tree.

The phylogenetic tree based on the 16S rRNA gene sequences shows that 5 strains of our study fall within 99.5% similarity with *A. ferrooxidans* AF465604 sequence. While Isolate HGM 30 was 98% similar to *A. ferrooxidans.*

In conclusion, these results provide evidence that the diversity among *A. ferrooxidans* isolates does not correlate with their geographical site of isolation. Since 16S rRNA sequence comparison showed 100% similarity between isolates SRD5 and 2MC, which are isolated from Ambamata polymetallic mine and Malanjkhand copper mine situated in two different states – Gujarat and Rajasthan. In the same way, 3 out of 4 isolates obtained from Hutti gold mine show more than 99% similarity, while the 4th isolate - HGM 30 is least similar to other *A. ferrooxidans* isolates.

However the results of RAPD and DNA analysis also do not match where isolate SRD 5 could be singled out as the most dissimilar strain out of all the strains tested. The the experimental finding in terms of physiological, metabolic and molecular profile gives a useful insight about minor details of characteristics of iron oxidizing organism *Acidithiobacillus ferrooxidans.* The development of fast growing, arsenic resistant SRD 5 strain is a major developing step towards large scale, short term leaching of arsenopyrite concentrate.

Fig. 7.48 : Phylogenetic tree showing the relationship between the 16S rRNA gene sequences of *Acidithiobacillus ferrooxidans* isolates from different eco-systems. The sequence data of other *Acidithiobacillus* type species were recovered from genebank and included in the tree. Phylogenic tree reconstruction analysis was done by UPGMA method. The bootstrap values from 1000 replications are shown at each of the branch points on the tree. The ruler at the bottom indicates % similarity.



1. Eight iron-oxidizing strains were obtained by isolation as well as selection from previously isolated and preserved cultures in the laboratory. The cultures were isolated from soil and water sample and they represented predominant mining microflora of Hutti gold mine, Ambamata polymetallic mine, Malanjkhand copper mine and Neyveli lignite mine.

2. Among eight iron oxidizing isolates, highest IOR of 0.570 g/L/h and SOR of 1.58 g/l/d was achieved with isolate SRD 5 and E' respectively, both of which were isolated from Ambamata mine, Gujarat. Isolate E', HGM 26 and 2MC showed good sulfur oxidation, while rest of the isolates showed lower sulfur oxidation. Isolate SRD 5, E' and 2MC could use thiosulfate as energy source, but isolate HGM 38 could not utilize thiosulfate at all.

3. The growth of isolates SRD 5, HGM 26 and HGM 38 was checked in presence of 11 inorganic substrates (ferrous sulfate, pyrite, elemental sulfur, tetrathionate, thiosulfate, thiocyanate, methanol, formaldehyde, sodium formate, methyl amine and methyl formate) and 3 metal sulphides (CuS, NiS and ZnS) as substrates to serve as sole energy sources. The isolates showed considerable difference in their response.

4. Thiosulfate supported the highest growth of culture SRD 5, which corresponded to 87.5 fold increase in cell count in 20 days. Sulfur supported the highest growth of HGM 26 and HGM 38. Using sulfur as energy source, cell count in the medium increased 100, 44 and 24 fold in 13 days for culture HGM 26, SRD 5 and HGM 38 respectively.

5. Pyrite was second best energy source for all 3 isolates, which gave 45.5, 42.5 and 22.5 fold increase in cell count in 13 days in isolate SRD 5, HGM 26 and HGM 38 respectively. None of the isolate tested could use the organic energy source.

6. When three metal sulfides namely - copper sulfide (CuS), nickel sulfide (NiS) and zinc sulfide (ZnS) were tested for their ability to serve as energy source, isolate SRD 5 could use both copper and nickel sulfides as sole source of energy and showed 24 and 22.5 fold increase in their presence respectively in 25 d. SRD5 showed very little growth in presence of ZnS.

7. HGM 26 and HGM 38 could use only copper sulfide as energy source and showed 16.8 and 14 fold increase in cell load in its presence in 18 d. HGM 26 and HGM 38 failed to use ZnS as source of energy.

8. Contrary to isolate SRD 5 and HGM 26, nickel sulphide served as better energy source for HGM 38 and gave 15.6 fold increase in cell count in 14 d. By using NiS as energy source, isolate HGM 26 gave only 5 fold increase in cell count.

9. Since mining bacteria are highly diverse in their metabolic requirement, various parameters like NaCl, PO_4 salt, growth in anaerobic condition, presence of organic media and higher concentration of thiosulfate was tested. Isolate HGM 26 and SRD 5 showed >99% oxidation of 1% thiosulfate, while isolate HGM 38 did not utilize 1% thiosulfate at all. No isolate could utilized 6% thiosulfate as energy source.

10. Presence of 4% phosphate in medium had little effect on iron oxidation, where 94.2 and 92.4% iron oxidation was completed by isolates HGM 26 and HGM 38 respectively. However isolate SRD 5 was affected more as it could oxidize only 71% ferrous iron.

11. Addition of 5% NaCl in medium showed adverse effect on activity of all the isolates, as 66%, 87% and 91% inhibition in iron oxidation was observed with SRD 5, HGM 26 and HGM 38 respectively. However isolate SRD 5 showed adaptation to high saline environment and better iron oxidation was seen after a lag period of 15 days.

12. Growth and ferrous iron oxidation activity of isolate SRD 5 was not inhibited in presence of 0.5% glucose, mannitol, sucrose or citric acid as more than 99% iron was oxidised in 48 h in their presence. Moreover presence of glucose and mannitol in the medium gave 1.8 and 1.13 fold increase in cell load respectively, as compared to ferrous sulphate alone, indicating that the isolate was capable of mixotrophic growth. The addition of fructose, yeast extract, TSB and malic acid gave 93% to 96% inhibition in iron oxidation.

13. At ferrous iron concentrations of 5% to 25% in the medium, addition of 10% ferric showed no increase in iron oxidation as compared to inoculum. Moreover, it was observed that ferric iron had marginal negative effect on iron oxidation. Moreover isolate was able to completely oxidize 5% to 20% ferrous sulfate in 2 to 10 days respectively, it could not oxidize 25% ferrous sulfate even after 35 days.

14. Hence it is understood that isolate SRD5 is able to completely resist 20% ferrous sulfate or 400 mM (Fe^{+2}), but in presence of 500 mM (Fe^{+2}), iron oxidation becomes very slow and almost 90% decrease in iron oxidation is observed.

15. When effect of arsenite (As^{+3}) and arsenate (As^{+5}) ions on activity of isolate SRD 5 and HGM 26 was checked, it was found out that isolate SRD5 was able to resist 20 mM to 100 mM arsenate ion (As^{+5}) added as potassium arsenate in its first exposure itself.

16. Upto 40 mM potassium arsenate in ferrous oxidation medium did not have any negative effect on iron oxidation and the iron oxidation in presence of 20 mM and 40 mM arsenate was similar to control flask. However, in presence of 60 mM As^{+5}, 13% decreased IOR was observed. In flasks containing 80 mM and 100 mM As^{+5}, 28% and 32% decreased in IOR was observed.

17. After >99% iron oxidation in presence of 100 mM As^{+5}, isolate SRD 5 was exposed to 100 mM to 1000 mM As^{+5}. This time isolate SRD 5 showed same iron oxidation rate in control flask as well as 100 mM As^{+5} containing flask. However in presence of 200 mM arsenate, only 39% IOR was noted as compared to control. Iron oxidation was inhibited at 300 mM potassium arsenate.

18. Decrease in IOR was observed at all concentration of potassium arsenite tested, which ranged from 20 to 100 mM As^{+3}. In presence of 20 mM and 40 mM arsenite, the iron oxidation rate decreased by 27% and 29% respectively as compared to control flask. The IOR achieved in presence of 80 mM and 100 mM As^{+3} were 114 and 89 mg/L/h respectively.

19. All the eight isolates were tested for tolerance to five metals and metalloids viz. copper sulfate ($CuSO_4.5H_2O$), zinc sulfate ($ZnSO_4.H_2O$), nickel sulfate ($NiSO_4.6H_2O$), cadmium sulfate ($3CdSO_4.8H_2O$) and potassium arsenate ($KAsO_2$).

20. In general all the isolates showed good resistance to 865 and 802 mM copper sulphate. The isolates showed 32 to 61% iron oxidation in presence of 802 mM copper, while 35 to 53% iron oxidation was observed in medium containing 865mM copper.

21. In presence of zinc sulphate, nickel sulphate, cadmium sulphate and potassium arsenate the isolates showed only 10-30% iron oxidation.

22. Isolate HGM 17 proved to be the most metal resistant isolate as it gave highest resistance against 802 mM copper, 1230 mM nickel, 498 mM cadmium and 84 mM arsenate.

23. HGM 26 was the only isolate which showed >99% iron oxidation in presence of both 84 mM and 40 mM As^{+5}. Along with iron oxidation, HGM 26 also showed 6 and 3.57 fold increase in cell load in presence of arsenate.

24. For microbial leaching of pyrite, both inorganic as well organic medium were compared for better oxidation. Isolate HGM 26 and isolate HGM 30 showed more solubilization of ferrous iron from pyrite in presence of organic supplement in medium.

25. Pyrite kinetics of three iron-oxidizing isolates was studied in 9K medium with addition of 0.05, 0.1, 0.25, 0.5, 1, 2 and 4% commercially available pyrite. While 65% to 86% oxidation was achieved in lower concentrations of pyrite (0.05 to 0.5%), only 35% to 48% leaching was observed at higher concentrations (1% to 4%) pyrite.

26. Among the four isolates studied, isolate HGM 26 gave highest oxidation of pyrite. The K_m value given by isolates SRD 5, HGM 30, HGM 26 and HGM 38 was 1, 0.35, 0.33 and 0.26 respectively. While V_{max} value for the same isolates was 1.17, 0.87, 0.35 and 1.32 g $Fe.L^{-1}.day^{-1}$ respectively.

27. During arsenopyrite kinetic study, in 0.05% & 0.1% concentration of arsenopyrite ore, more than 99% leaching could be achieved. While 0.25% PD showed 89% extraction. 0.5, 1 and 2% ore gave considerable fair leaching, which was 59%, 41% and 35% respectively in the first exposure itself.

28. During study of diversity by RAPD analysis, when all the RAPD bands obtained by each primer were put together to compare the similarity and differences between the isolates, isolates E' and HGM 17 were found to be most similar among all isolates tested eventhough these two isolates belong to two different geographical location and different mining environments. As against this, isolate SRD 5 which has been isolated from Amabamata polymetallic mine was found to be most dissimilar of all.

29. 16S rRNA sequence comparison showed 100% similarity between isolates SRD 5 and 2 MC, which are isolated from Ambamata

polymetallic mine and Malanjkhand copper mine situated in two different states – Gujarat and Rajasthan. These results provide evidence that the diversity among *A. ferrooxidans* isolates does not correlate with their geographical site of isolation.

30. Finally, the experimental finding in terms of physiological, metabolic and molecular profile gives a useful insight about minor details of characteristics of iron oxidizing organism *Acidithiobacillus ferrooxidans*. The development of fast growing, arsenic resistant SRD 5 strain is a major developing step towards large scale, short term leaching of arsenopyrite concentrate.

1. Silverman and Lundgren (9K) Medium

$(NH_4)_2SO_4$	3.0 g
KCl	0.1 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.5 g
$Ca(NO_3)_2$	0.01 g
Distilled water	700 ml
10 N H_2SO_4	1.0 ml
$FeSO_4.7H_2O$ (14.74% w/v solution)	300 ml

The basal salts and iron solutions are sterilized separately by autoclaving and filtration respectively and combined when basal salt medium cools down. The pH of medium is set between 2.8 to 3.0.

The above medium was modified during the investigations in terms of energy source $FeSO_4.7H_2O$ concentration. In place of 300 ml solution of 14.74% concentration, 100 ml solution of 20% concentration was added in 900 ml basal salt medium to obtain 2% ferrous sulfate concentration.

2. TSB medium

$(NH_4)_2SO_4$	1.25 g
$MgSO_4.7H_2O$	0.5 g
TSB	0.5 g
K_2HPO_4	0.02 g
pH (adjusted with 10% H_2SO_4)	2.0±0.2

3. Basal mineral salts medium (S_0)

Na_2HPO_4	1.2g
KH_2PO_4	1.8 g
$MgSO_4.7H_2O$	0.1 g
$(NH_4)_2SO_4$	0.1 g
$CaCl_2$	0.03 g
$FeCl_3.6H_2O$	0.02 g
$MnS0_4.4H_2O$	0.02 g
Distilled water	1000 ml

3.1 S_5 medium

Basal medium (S_0)	1000 ml	(without Na_2HPO_4)
KH_2PO_4	2.0 g	(as the only phosphate)
NaCl	1.0 g	(to correct the ionic balance)

Thiosulfate/Sulfur/Iron was added to above basal salt medium and pH was adjusted to 4.6, 3.5 or 2.7 respectively.

3.2 S_6 medium

Basal medium (S_0)	1000 ml
$Na_2S_2O_3.5H_2O$	10.0 g
pH	4.6

3.3 S_7 medium

Basal medium (S_0)	1000 ml
NH_4CNS	0.02 g

3.4 S_8 medium

Used for the *T. denitrificans, T. intermedius* and *T.thermophilica*

Basal medium (S_0)	1000 ml
$Na_2S_2O_3.5H_2O$	10.0 g
$NaHCO_3$	0.5 g/l
KNO_3	5.0 g/l
pH	4.6

Medium was filled upto the rim in screwcap bottles. When gas formation was observed in the cultures, two-third of the culture medium was replaced aseptically with fresh medium.

1. PURIFICATION

Purification of the isolate was done by serial dilution tube culture method. A series of 15 tubes were made having 9 ml 9K medium and sterilized by autoclaving.

The culture to be purified was grown in iron containing media. One ml inoculum from 48 h old culture was inoculated in first tube and mixing was done by shaking for 1 minute on vortex mixer.

Serial dilution of the inoculum was continued similarly with intermittent vortexing till 15^{th} tube and tubes were incubated at static condition at 30 ± 2 °C for 15 days.

The highest dilution tube showing iron oxidation after 15 days was considered to be a pure culture.

2. CULTURE HARVESTING

Culture growing on ferrous iron logarithmic phase was taken and vacuum filtration was done using 0.22 μ nylon membrane filter (Pall lifesciences).

The iron precipitates along with cells filtered on membrane were suspended in 100 ml chilled sterile acidified distilled water. The contents were shaken vigorously in a 250 ml reagent bottle for 1 minute and allowed to stand in refrigerator for 5-6 hrs.

During this time iron precipitates settled at bottom of the bottle in form of thin layer, and cells remained suspended in supernatant fluid making it a turbid solution.

The supernatant fluid was carefully withdrawn with help of HiMedia automated pipette and transferred to sterile centrifuge tubes.

Cells were centrifuged at 12000 rpm for 15 minutes in Remi cooling ultracentrifuge.

Cell pellet obtained after centrifugation was washed twice with cold acidified distilled water and finally pallet was resuspended in pH 2 distilled water.

Cell suspension so obtained was standardized by counting the cell number in Neubaur's chamber and adjusting the cell load as required in the experiment.

The harvested cells could be stored in refrigerator at 5 °C for 5-6 days successfully without any noticeable loss of iron oxidation activity.

3. DETERMINATION OF FERROUS IRON

3.1 REAGENTS

(A) Standard ferrous solution

Weigh out accurately 14.0 g of ferrous sulfate crystals, dissolve in 450 ml of 5% (v/v) sulfuric acid in a 500 ml volumetric flask and make upto the mark with distilled water. Shake well, titrate 25 ml with the standard 0.1 N potassium dichromate using diphenylamine as internal indicator.

(B) Standard 0.1 N potassium dichromate

Weigh accurately 4.9 g of potassium dichromate and transfer the salt quantitatively to a 1 liter measuring flask. Dissolve salt completely in water in the flask and make the volume upto the mark. Shake well.

Calculate exact normality by dividing the actual weight of the potassium dichromate employed by the theoretical weight for 1 liter of normal solution (49.035 g). An exactly 0.1 N solution may be prepared by weighing out 4.904 g of the salt and dissolving in water and diluting to 1 liter in a volumetric flask.

(C) Diphenylamine indicator (1%)

Weigh 1.0 g diphenylamine indicator and dissolve in 100 ml of concentrated sulfuric acid.

3.2 PROCEDURE

Take 200 ml of 2.5% (v/v) sulfuric acid followed by 5 ml of 85% orthophosphoric acid. Add 3 drops of indicator and titrate slowly with the standard dichromate, while stirring constantly until the solution assumes a bluish-green tint near the end point. Continue the titration, adding the dichromate solution dropwise and maintaining an interval of a few seconds between each drop, until the addition of 1 drop causes the formation of an intense purple or violet blue colouration, which remains permanent after shaking and is unaffected on further addition of the dichromate. Carry out two or at the most three titrations, which should agree within 0.1 ml.

Use of phosphoric acid lowers the oxidation potential of the ferric-ferrous system by following a complex $Fe(HPO_4)^+$ with the ferric ions so that the equivalence potential coincides more nearly with that of the indicator.

The action of diphenylamine as an indicator depends upon its oxidation first into colourless diphenylbenzidine, which is the red indicator and is reversibly further oxidized to diphenylbenzidine violet.

Diphenylbenzidine violet undergoes further oxidation if allowed to stand with excess of dichromate solution, the excess oxidation is irreversible and red or yellow products of unknown compositions are produced.

1 ml 0.1 N $K_2Cr_2O_7$ = 5.585 mg Fe^{+2}

4. DETERMINATION OF TOTAL IRON BY 1-10 PHENANTHROLINE SPECTROPHOTOMETRIC METHOD

4.1 REAGENTS

(A) Stock iron solution

Slowly add 20 ml of concentrated H_2SO_4 to 50 ml distilled water and dissolve 1.404 g of ferrous ammonium sulfate ($Fe(NH_4)_2(SO_4)_2.6H_2O$). Add 0.1 ml potassium permanganate dropwise until a faint pink colour persists. Dilute to 1000 ml and mix. **1 ml = 200 μg Fe**

(B) Standard iron solution

Pipette 50 ml stock solution into 1000 ml volumetric flask and dilute to the mark with distilled water. **1 ml = 10 μg Fe**

(C) Hydroxylamine solution

Dissolve 10 g $NH_2OH.HCl$ in 1000 ml distilled water.

(D) Ammonium acetate buffer

Dissolve 250 g ammonium acetate ($NH_4C_2H_3O_2$) in 150 ml distilled water. Add 700 ml concentrated glacial acetic acid. Final volume will be slightly more than 1000 ml.

(E) 1-10 phenanthroline solution

Dissolve 100 mg of 1-10 phenanthroline monohydrate ($C_{12}H_8N_2.H_2O$) in 1000 ml distilled water by stirring and heating to 80°C. Do not boil. Discard the solution if it darkens. Heating is unnecessary if 2 drops of concentrated HCl is added.

4.2 PROCEDURE

Dilute the sample to bring iron in it within range of 20-200 μg/ml. Take 1 ml of this sample in 50 ml volumetric flask and add distilled water upto 50ml mark. Transfer the content in 50 ml glass beaker and add 2 ml

concentrated HCl and 1 ml hydroxylamine solution. Add glass beads in it and heat the solution on a hot plate till 25 ml solution remains.

Cool to room temperature and transfer it again into at 50 ml volumetric flask. Add 10 ml ammonium acetate buffer solution and 4 ml phenanthroline solution in it. Mix it thoroughly and add distilled water upto 50 ml mark. Again mix it and allow to stand for 10-15 min. for maximum colour development. Measure optical density at 520 nm and compare the reading with standard graph of Optical density (O.D.) 10-100 μg Fe.

5. DETERMINATION OF THIOSULPHATE BY TITRIMETRIC METHOD USING STANDARD IODINE SOLUTION

5.1 REAGENTS

(A) Standard iodine solution

Dissolve 20 g of iodate free potassium iodide in 30-40 ml of water in a glass stoppered 1 litre flask. Weigh out 12.7 g of analytical grade iodine on a watch glass on balance and transfer it by means of a dry funnel into the concentrated potassium iodide solution. Insert the glass stopper into the flask and shake till all the iodine is dissolved. Make up to the mark with distilled water.

12.691 g iodine per litre = 0.1 N iodine

5.2 PROCEDURE

Titrate the solution of 0.1 N iodine with sample of sodium thiosulphate solution from a burette. When the liquid is pale yellow in colour, add 2 ml of starch solution and continue titration dropwise until the blue colour is just destroyed.

Calculate the concentration of thiosulphate by following formula.

0.12691 g iodine = 1 ml 1 N $Na_2S_2O_3$

6. ESTIMATION OF SULFATE

6.1 REAGENTS

(A) Conditioning reagents

Mix 50 ml glycerol with a solution containing 30 ml concentrated HCl, 300 ml distilled water, 100 ml 95% ethyl alcohol and 75 g NaCl.

(B) Barium chloride solution (1%)

Mix 1 g pure barium chloride crystals in 100 ml ion free distilled water.

(C) Standard sulfate solution

Dissolve 147.9 mg anhydrous Na_2SO_4 and dilute to 1000 ml.

1 ml = 100 μg SO_4

6.2 PROCEDURE

Take suitable volume of sample in 250 ml conical flask and dilute to 100 ml. Add 5 ml of conditioning reagent accurately and mix well. Keep the flask constantly stirred with the help of stirrer. Add 1 ml barium chloride solution in it and continue stirring. Measure the turbidity developed after every 30 second for 2 minutes on spectrophotometer at 420 nm. After 2 minutes stirring, reading will become constant. Note this reading for calculation purpose.

Prepare standard curve with known concentrations of standard sulphate solution with above-mentioned procedure. Calculate concentration of sulfate present in the sample from standard curve.

7. BACTERIAL GENOMIC DNA ISOLATION USING CTAB METHOD.

- Pellet containing bacterial cells is suspended in 250 µl Tris-EDTA (pH-8.0), 250 µl SDS (10% w/v) and 10 µl of proteinase K solution (20mg/ml) and incubated for 1 h at 37^0C.
- Subsequently, 500 µl of 5M NaCl followed by 100 µl CTAB (10% solution in 0.7M NaCl) was added and incubated in water bath for 10 min at 65^0C.
- The solution was centrifuged at 8,000 rpm for 10 min after mixing with equal volume of chloroform: isoamyl alcohol (24:1)
- Upper phase obtained after centrifugation was transferred to clean microfuge tube.
- Equal volume of phenol : chloroform : isoamyl alcohol (25:24:1) was added, mixed well by inverting, spun for 10 min at 10,000 rpm and upper aqueous phase was transferred again to a clean microfuge tube.
- In the collected supernatant, the DNA is precipitated with one-tenth volume of ammonium acetate (7.5M) and double the volume of chilled absolute ethanol.
- Tube containing DNA, ammonium acetate and absolute ethanol was centrifuged for 10 min at 11,000 rpm and supernatant was discarded.
- The pellet was washed in 70% ethanol and again spun for 5 min at 11,000 rpm.
- Ethanol is discarded and pellet was dried.
- DNA is resuspended in 200 µl sterile distilled water and incubated in water bath at 65 °C for one hour and stored at -20 °C till use.

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A. ORAL PRESENTATIONS

Regional conference, Department of Microbiology, School of Sciences, Gujarat University, Ahmedabad. March 2, 2009. **(First prize)**

METABOLIC AND PHYSIOLOGICAL STUDIES OF SELECTED IRON OXIDIZERS

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Abstract

Due to the limited type of substrates available in mining environments and prevailing extreme conditions, the biotopes were expected to be extremely poor in diversity of their microbial flora. However the mining environment show a great microbial diversity with at least 11 prokaryotic divisions living at AMD sites. Among these bacteria the most predominant one, *Acidithiobacillus sp.* is endowed with a remarkably broad metabolic capacity viz. oxidation of reduced iron and sulphur compounds, molecular hydrogen, formic acid and other metal ions. *At. ferrooxidans* plays an important role in iron (Fe^{+2}) oxidation. Hence differential characteristics of various iron oxidising autotrophs were studied in presence of different kinds of organic and inorganic substrates. The substrate studied included Ferrous ions, sulphur, thiosulphate, tetrathionate, thiocynate, pyrite, various metal sulphides and organic substrates like methanol, formaldehyde, sodium formate etc. Sulphur supported the highest growth of HGM 26 and HGM 38 giving 91 and 52 fold increase in cell count in 18 days respectively, while highest growth of SRD 5 was observed with thiosulphate, which gave 50-fold increase in cell number in 20 days. In presence of thiosulphate, continuous decrease in pH was observed in HGM 26 from 4.56 to 2.78 in 13 days, after which organism showed increase in cell count. On the other side, HGM 38 could not use thiosulphate at all. Pyrite supported the second highest growth in all 3

cultures, where 60, 24 and 18.5 fold increase in cell growth was observed in HGM 38, HGM 26 and SRD 5 respectively.

Among metal sulphides, copper sulphide acted as sole energy source for all the 3 cultures and gave 9 to 18 fold increase in growth in 20 days. Though Nickle sulphide supported growth of SRD 5, it showed no remarkable increase in growth of HGM 26 and HGM 38. No organic substrate supported the growth of any of the isolate studied. These results illustrate the diverse metabolic activity in iron oxidizers and selective growth pattern given by different energy sources.

VIGNAN PARISHAD: SCIENCE EXCELLENCE – 2011 (First prize)

Department of Botany, Gujarat University, Ahmedabad. January 8, 2011

CHARACTERIZATION OF VARIOUS *Acidithiobacillus ferrooxidans* STRAINS ISOLATED FROM VARIOUS MINES ACROSS INDIA, USING RANDOM AMPLIFIED POLYMORPHIC DNA AND 16S rRNA GENE ANALYSIS

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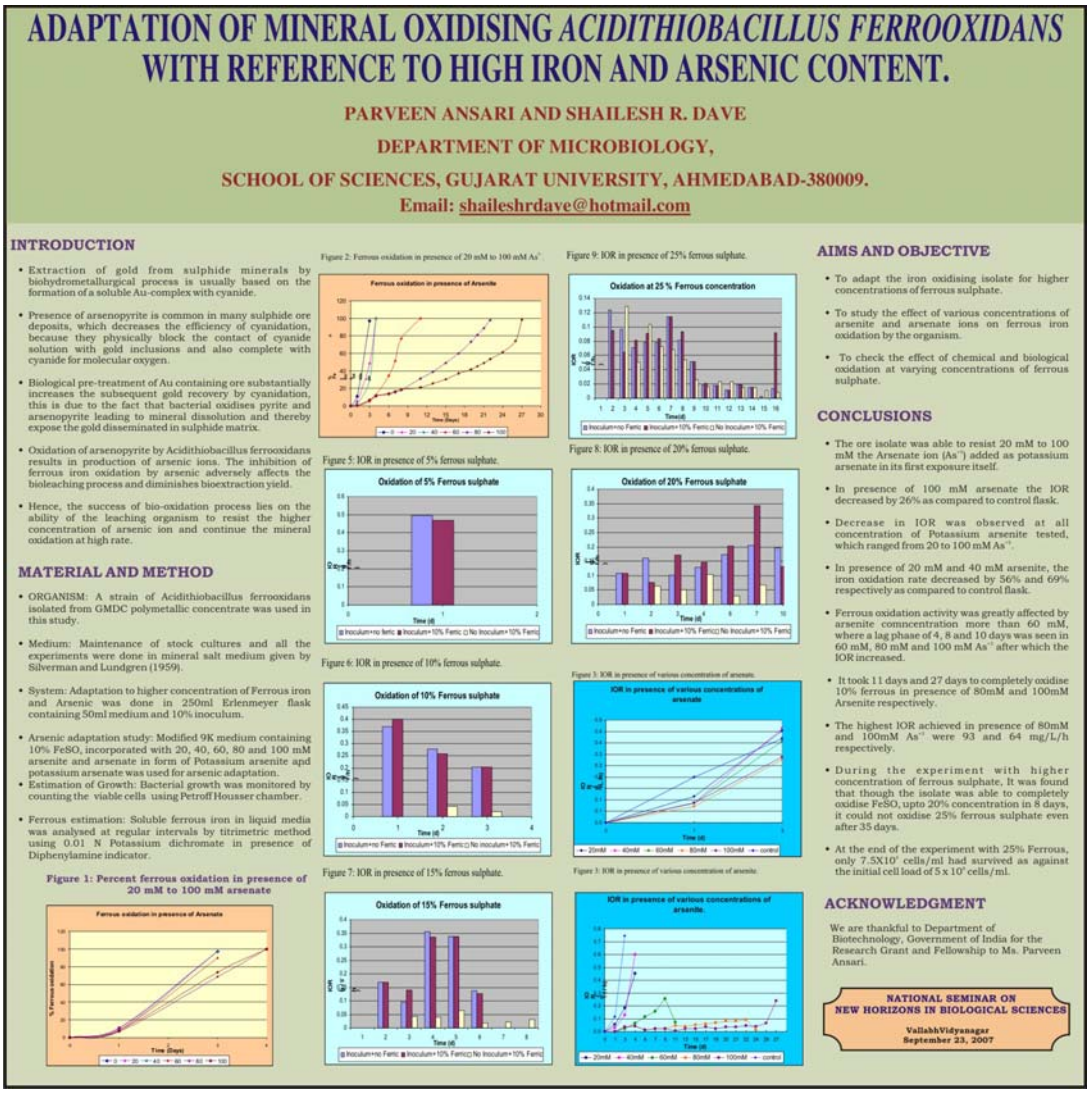
Abstract

Acidophilic, chemoautotrophic, iron and sulphur oxidizing bacteria are an integral component of commercial bioleaching processes. *Acidithiobacillus ferrooxidans* is considered to be the most important biological catalyst in it. This species is easily detected by their ability to oxidize ferrous iron resulting in generation of rust colour of ferric ions combined with rod shaped cell morphology which differentiates it from *Leptospirillum ferrooxidans,* but high level of diversity exists among its various isolates. Eight strains of *A. ferrooxidans* SRD 5, HGM 17, HGM 26, HGM 30, HGM 38, E', 2MC and Lig-Tf were obtained from various mine sites across India. These isolates were identified as *A. ferrooxidans* by phenotypic characterization and 16S rRNA sequence analysis. In this study, phylogenetic analysis of 16S rRNA fragments combined with Random Amplified Polymorphic DNA (RAPD) analysis has proved to be successful in differentiating various strains of *A. ferrooxidans*. RAPD profile of genomic DNA extracted from these isolates showed that banding pattern obtained by each primer showed more similarity among some isolates making it a group, as compared to others and these similar isolates made different groups with each new primer used. The genomic banding profile of SRD 5 was significantly different from that of others.

B. POSTER PRESENTATIONS

NATIONAL SEMINAR ON NEW HORIZONS IN BIOLOGICAL SCIENCES

N.V. Patel College of Pure and Applied Sciences, Vallabh Vidyanagar, Gujarat, India. September 23, 2007. **(Second prize)**



ADAPTATION OF MINERAL OXIDISING *ACIDITHIOBACILLUS FERROOXIDANS* WITH REFERENCE TO HIGH IRON AND ARSENIC CONTENT.

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INTRODUCTION

- Extraction of gold from sulphide minerals by biohydrometallurgical process is usually based on the formation of a soluble Au-complex with cyanide.
- Presence of arsenopyrite is common in many sulphide ore deposits, which decreases the efficiency of cyanidation, because they physically block the contact of cyanide solution with gold inclusions and also complete with cyanide for molecular oxygen.
- Biological pre-treatment of Au containing ore substantially increases the subsequent gold recovery by cyanidation, this is due to the fact that bacterial oxidises pyrite and arsenopyrite leading to mineral dissolution and thereby expose the gold disseminated in sulphide matrix.
- Oxidation of arsenopyrite by Acidithiobacillus ferrooxidans results in production of arsenic ions. The inhibition of ferrous iron oxidation by arsenic adversely affects the bioleaching process and diminishes bioextraction yield.
- Hence, the success of bio-oxidation process lies on the ability of the leaching organism to resist the higher concentration of arsenic ion and continue the mineral oxidation at high rate.

MATERIAL AND METHOD

- ORGANISM: A strain of Acidithiobacillus ferrooxidans isolated from GMDC polymetallic concentrate was used in this study.
- Medium: Maintenance of stock cultures and all the experiments were done in mineral salt medium given by Silverman and Lundgren (1959).
- System: Adaptation to higher concentration of Ferrous iron and Arsenic was done in 250ml Erlenmeyer flask containing 50ml medium and 10% inoculum.
- Arsenic adaptation study: Modified 9K medium containing 10% FeSO, incorporated with 20, 40, 60, 80 and 100 mM arsenite and arsenate in form of Potassium arsenite and potassium arsenate was used for arsenic adaptation.
- Estimation of Growth: Bacterial growth was monitored by counting the viable cells using Petroff Hausser chamber.
- Ferrous estimation: Soluble ferrous iron in liquid media was analysed at regular intervals by titrimetric method using 0.01 N Potassium dichromate in presence of Diphenylamine indicator.

Figure 1: Percent ferrous oxidation in presence of 20 mM to 100 mM arsenate

Figure 2: Ferrous oxidation in presence of 20 mM to 100 mM As^{3+}.

Figure 5: IOR in presence of 5% ferrous sulphate.

Figure 6: IOR in presence of 10% ferrous sulphate.

Figure 7: IOR in presence of 15% ferrous sulphate.

Figure 9: IOR in presence of 25% ferrous sulphate.

Figure 8: IOR in presence of 20% ferrous sulphate.

Figure 3: IOR in presence of various concentration of arsenate.

Figure 3: IOR in presence of various concentration of arsenite.

AIMS AND OBJECTIVE

- To adapt the iron oxidising isolate for higher concentrations of ferrous sulphate.
- To study the effect of various concentrations of arsenite and arsenate ions on ferrous iron oxidation by the organism.
- To check the effect of chemical and biological oxidation at varying concentrations of ferrous sulphate.

CONCLUSIONS

- The ore isolate was able to resist 20 mM to 100 mM the Arsenate ion (As^{+5}) added as potassium arsenate in its first exposure itself.
- In presence of 100 mM arsenate the IOR decreased by 26% as compared to control flask.
- Decrease in IOR was observed at all concentration of Potassium arsenite tested, which ranged from 20 to 100 mM As^{+3}.
- In presence of 20 mM and 40 mM arsenite, the iron oxidation rate decreased by 56% and 69% respectively as compared to control flask.
- Ferrous oxidation activity was greatly affected by arsenite comncentration more than 60 mM, where a lag phase of 4, 8 and 10 days was seen in 60 mM, 80 mM and 100 mM As^{+3} after which the IOR increased.
- It took 11 days and 27 days to completely oxidise 10% ferrous in presence of 80mM and 100mM Arsenite respectively.
- The highest IOR achieved in presence of 80mM and 100mM As^{+3} were 93 and 64 mg/L/h respectively.
- During the experiment with higher concentration of ferrous sulphate, It was found that though the isolate was able to completely oxidise FeSO, upto 20% concentration in 8 days, it could not oxidise 25% ferrous sulphate even after 35 days.
- At the end of the experiment with 25% Ferrous, only 7.5X10^7 cells/ml had survived as against the initial cell load of 5 x 10^7 cells/ml.

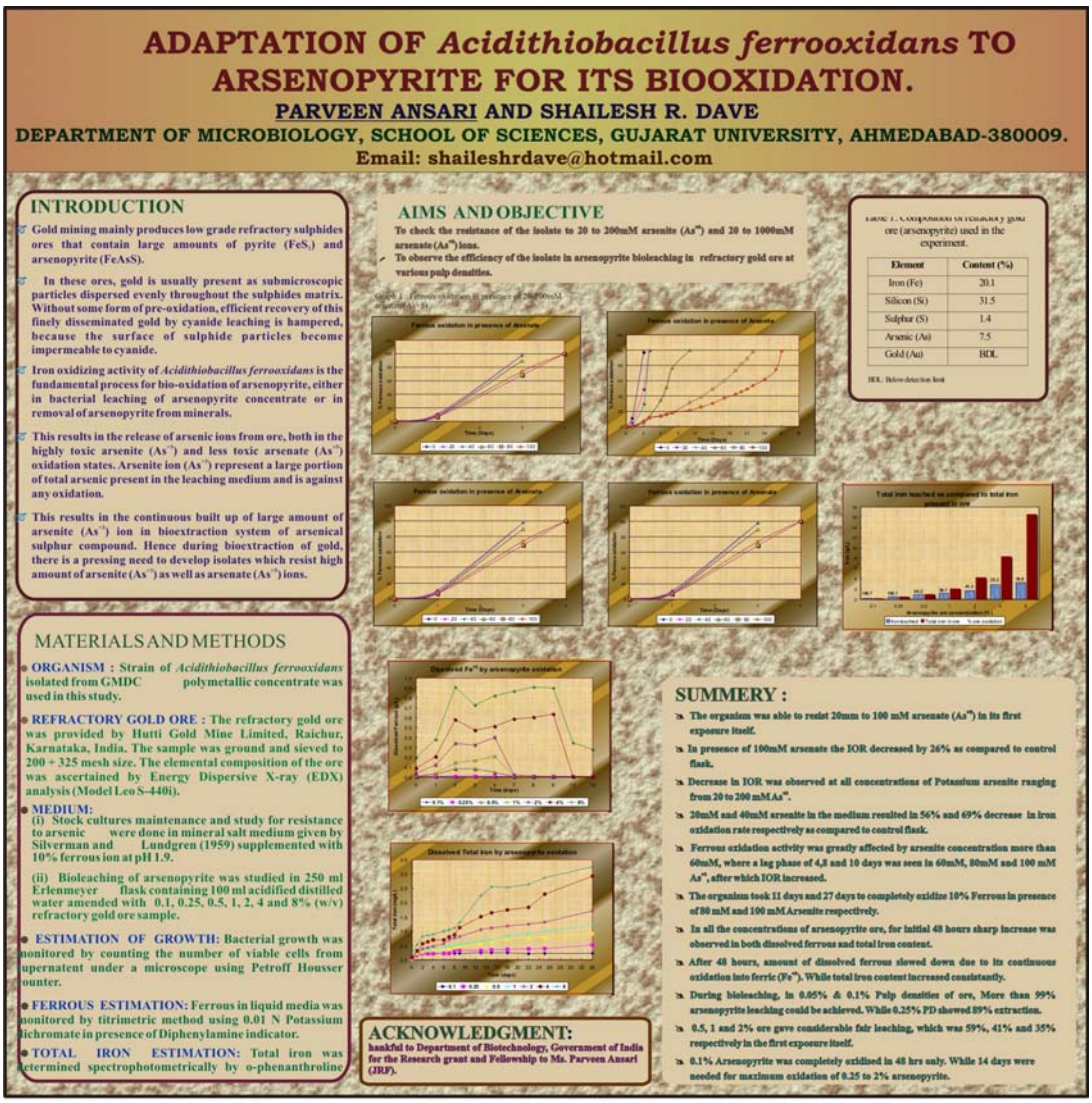
ACKNOWLEDGMENT

We are thankful to Department of Biotechnology, Government of India for the Research Grant and Fellowship to Ms. Parveen Ansari.

NATIONAL SEMINAR ON NEW HORIZONS IN BIOLOGICAL SCIENCES
VallabhVidyanagar
September 23, 2007

48th ANNUAL CONFERENCE OF ASSOCIATION OF MICROBIOLOGISTS OF INDIA

Indian Institute of Technology, Chennai. December 18-21, 2007



ADAPTATION OF *Acidithiobacillus ferrooxidans* TO ARSENOPYRITE FOR ITS BIOOXIDATION.

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INTRODUCTION

- Gold mining mainly produces low grade refractory sulphides ores that contain large amounts of pyrite (FeS_2) and arsenopyrite (FeAsS).
- In these ores, gold is usually present as submicroscopic particles dispersed evenly throughout the sulphides matrix. Without some form of pre-oxidation, efficient recovery of this finely disseminated gold by cyanide leaching is hampered, because the surface of sulphide particles become impermeable to cyanide.
- Iron oxidizing activity of *Acidithiobacillus ferrooxidans* is the fundamental process for bio-oxidation of arsenopyrite, either in bacterial leaching of arsenopyrite concentrate or in removal of arsenopyrite from minerals.
- This results in the release of arsenic ions from ore, both in the highly toxic arsenite (As^{+3}) and less toxic arsenate (As^{+5}) oxidation states. Arsenite ion (As^{+3}) represent a large portion of total arsenic present in the leaching medium and is against any oxidation.
- This results in the continuous built up of large amount of arsenite (As^{+3}) ion in bioextraction system of arsenical sulphur compound. Hence during bioextraction of gold, there is a pressing need to develop isolates which resist high amount of arsenite (As^{+3}) as well as arsenate (As^{+5}) ions.

AIMS AND OBJECTIVE

To check the resistance of the isolate to 20 to 200mM arsenite (As^{+3}) and 20 to 1000mM arsenate (As^{+5}) ions.
To observe the efficiency of the isolate in arsenopyrite bioleaching in refractory gold ore at various pulp densities.

Table 1: Composition of refractory gold ore (arsenopyrite) used in the experiment.

Element	Content (%)
Iron (Fe)	20.1
Silicon (Si)	31.5
Sulphur (S)	1.4
Arsenic (As)	7.5
Gold (Au)	BDL

BDL: Below detection limit

MATERIALS AND METHODS

- **ORGANISM :** Strain of *Acidithiobacillus ferrooxidans* isolated from GMDC polymetallic concentrate was used in this study.
- **REFRACTORY GOLD ORE :** The refractory gold ore was provided by Hutti Gold Mine Limited, Raichur, Karnataka, India. The sample was ground and sieved to 200 + 325 mesh size. The elemental composition of the ore was ascertained by Energy Dispersive X-ray (EDX) analysis (Model Leo S-440i).
- **MEDIUM:**
  (i) Stock cultures maintenance and study for resistance to arsenic were done in mineral salt medium given by Silverman and Lundgren (1959) supplemented with 10% ferrous ion at pH 1.9.
  (ii) Bioleaching of arsenopyrite was studied in 250 ml Erlenmeyer flask containing 100 ml acidified distilled water amended with 0.1, 0.25, 0.5, 1, 2, 4 and 8% (w/v) refractory gold ore sample.
- **ESTIMATION OF GROWTH:** Bacterial growth was monitored by counting the number of viable cells from supernatant under a microscope using Petroff Housser counter.
- **FERROUS ESTIMATION:** Ferrous in liquid media was monitored by titrimetric method using 0.01 N Potassium dichromate in presence of Diphenylamine indicator.
- **TOTAL IRON ESTIMATION:** Total iron was determined spectrophotometrically by o-phenanthroline

SUMMERY :

- The organism was able to resist 20mm to 100 mM arsenate (As^{+5}) in its first exposure itself.
- In presence of 100mM arsenate the IOR decreased by 26% as compared to control flask.
- Decrease in IOR was observed at all concentrations of Potassium arsenite ranging from 20 to 200 mM As^{+3}.
- 20mM and 40mM arsenite in the medium resulted in 56% and 69% decrease in iron oxidation rate respectively as compared to control flask.
- Ferrous oxidation activity was greatly affected by arsenite concentration more than 60mM, where a lag phase of 4,8 and 10 days was seen in 60mM, 80mM and 100 mM As^{+3}, after which IOR increased.
- The organism took 11 days and 27 days to completely oxidize 10% Ferrous in presence of 80 mM and 100 mM Arsenite respectively.
- In all the concentrations of arsenopyrite ore, for initial 48 hours sharp increase was observed in both dissolved ferrous and total iron content.
- After 48 hours, amount of dissolved ferrous slowed down due to its continuous oxidation into ferric (Fe^{+3}). While total iron content increased consistantly.
- During bioleaching, in 0.05% & 0.1% Pulp densities of ore, More than 99% arsenopyrite leaching could be achieved. While 0.25% PD showed 89% extraction.
- 0.5, 1 and 2% ore gave considerable fair leaching, which was 59%, 41% and 35% respectively in the first exposure itself.
- 0.1% Arsenopyrite was completely oxidised in 48 hrs only. While 14 days were needed for maximum oxidation of 0.25 to 2% arsenopyrite.

ACKNOWLEDGMENT:

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INTERNATIONAL SYMPOSIUM ON MICROBIAL BIOTECHNOLOGY: DIVERSITY, GENOMICS AND METAGENOMICS.

49th Annual conference of Association of Microbiologists of India. University of Delhi, New Delhi, November 18-20, 2008

SUBSTRATE UTILIZATION AND KINETIC STUDIES OF THREE ACIDOPHILIC IRON OXIDISERS

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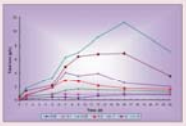
INTRODUCTION

- Analysis of the bacterial population on the surface of sulphidic minerals and in the solution suggested that the mineral oxidation is dependent largely on the fraction of bacteria, which is bound to mineral surface. In resent years the biochemical fundamentals of the leaching reactions have been the subject of intensive research.
- Mineral oxidising bacteria are highly diverse in terms of their physiologies and phylogenetic relationships. The dimension of the leaching chiefly resides on the strain and characteristic of the leaching bacteria.
- Due to their importance in the chemistry of mineral leaching, a significant amount of research activity has been focused on characterization of biohydrometallurgically important bacteria.
- In the present work, three chemoautotrophic biomining bacteria isolated from different mining ecosystems were studied for their substrate utilization efficiency and pyrite oxidizing kinetics.

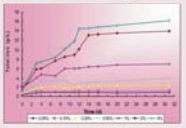
AIM AND OBJECTIVE

1. To compare substrate utilization efficiency of three iron oxidising isolates.
2. To observe the effects of various energy sources on the isolates.
3. To study pyrite oxidation at various pulp densities by all the three isolates.

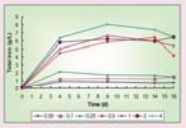
Total solubilized iron from pyrite by SRD 3



Total solubilized iron from pyrite by HGM 26



Total solubilized iron from pyrite by HGM 38



MATERIALS AND METHODS

ORGANISM

Three acidophilic chemoautotrophic isolates were studied. Among them SRD3 was isolated from polymetallic concentrate of Ambamata mine, Gujarat and HGM 26 and HGM 38 were isolated from samples from Hutti gold mine, Karnataka.

MEDIUM

(i) SUBSTRATE UTILIZATION STUDY: Study of various energy sources were done in mineral salt medium (9K) given by Silverman and Lundgren (1959) at pH 1.9.This medium (9K without $FeSO_4$) was amended with Pyrite, Sulphur, Thiosulphate, Thiocyanate, Tetrathionate at 1% (w/v) concentration.

(ii) PYRITE OXIDATION STUDY: Bioleaching of pyrite was studied in 9K medium without $FeSO_4$ amended with 0.05, 0.1, 0.25, 0.5, 1, 2 and 4 % (w/v) commercially available pyrite.

DETERMINATION OF GROWTH

Bacterial growth was monitored by counting the viable cells from supernatent under compound microscope using Petroff-Housser counter.

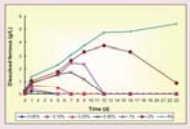
FERROUS IRON ESTIMATION

Soluble ferrous iron was monitored titrimetrically using 0.01 N $K_2Cr_2O_7$ and diphenylamine indicator.

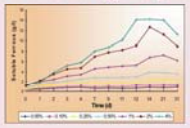
TOTAL IRON ANALYSIS

Total iron was determined spectrophotometrically by o-phenanthroline method using UV-Vis (Systronic 119) spectrophotometer.

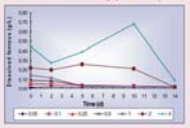
Solubilized ferrous from pyrite by SRD3



Solubilized ferrous from pyrite by HGM 26



Solubilized ferrous from pyrite by HGM 38

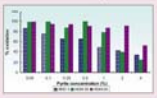


AMI - NEW DELHI 2008

Kinetic coefficients for pyrite oxidation by 3 iron oxidisers

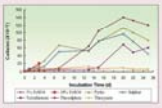
Isolate	Ks ($g\ Fe.L^{-1}$)	Vm ($g\ Fe.L^{-1}.day^{-1}$)
SRD 3	0.91	1.27
HGM 26	1.00	1.57
HGM 38	0.26	1.09

Percent pyrite oxidation by 3 isolates

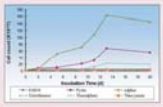


Cell count profiles during growth on six energy sources

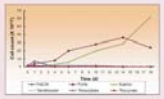
(i) SRD 3



(ii) HGM 26



(iii) HGM 38



CONCLUSION

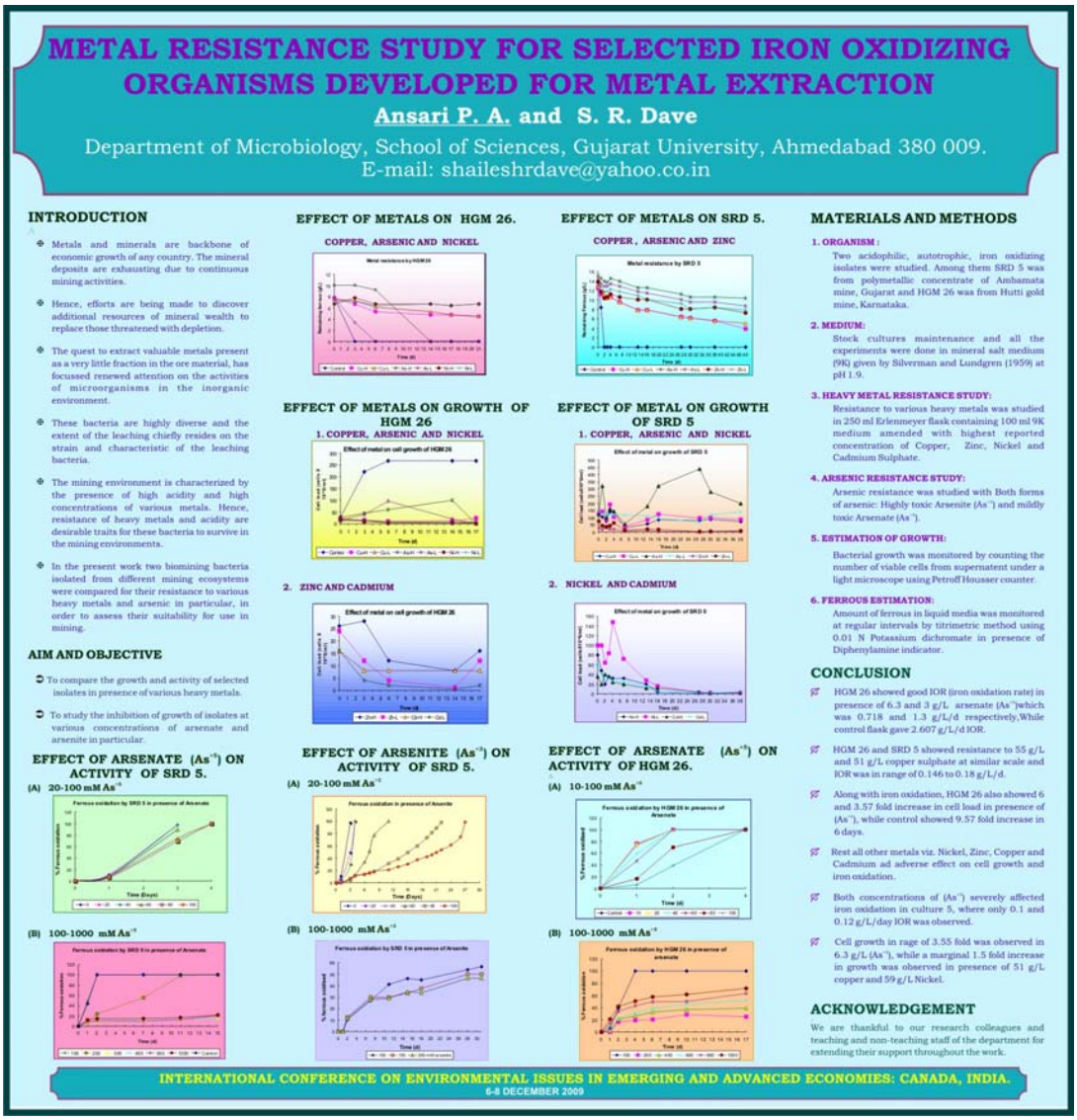
1. HGM 26 was found to be the best among the three isolates for pyrite oxidation followed by HGM 38. SRD 3 gave the least oxidation of pyrite.
2. Sulphur supported the highest growth of HGM 26 and HGM 38. Using sulphur as energy source, cell count in the medium increased 100, 44 and 24 fold in 13 days for culture HGM 26, SRD 3 and HGM 38 respectively.
3. In presence of sulphur, cell count of HGM 26 still increased after 13 days and reached upto 52 fold in18 days, while for SRD 3 it reached 60 fold in 20 days.
4. Thiosulphate supported the highest growth of culture SRD 3, which corresponded to 87.5 fold increase in cell count in 20 days.
5. Pyrite was second best energy source for all 3 isolates, which gave 60, 45.5 and 24 fold increase in cell count in 13 days in cultures HGM 38, SRD 3 and HGM 26 respectively.

ACKNOWLEDGEMENT

We are thankful to Department of Biotechnology, Government of India for research grant and fellowship to Ms. Parveen Ansari (JRF).

INTERNATIONAL CONFERENCE ON ENVIRONMENTAL ISSUES IN EMERGING AND ADVANCED ECONOMIES: CANADA, INDIA.

Department of Chemistry, School of Sciences, Gujarat University, Ahmedabad. December 6-8, 2009



METAL RESISTANCE STUDY FOR SELECTED IRON OXIDIZING ORGANISMS DEVELOPED FOR METAL EXTRACTION

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INTRODUCTION

- Metals and minerals are backbone of economic growth of any country. The mineral deposits are exhausting due to continuous mining activities.
- Hence, efforts are being made to discover additional resources of mineral wealth to replace those threatened with depletion.
- The quest to extract valuable metals present as a very little fraction in the ore material, has focussed renewed attention on the activities of microorganisms in the inorganic environment.
- These bacteria are highly diverse and the extent of the leaching chiefly resides on the strain and characteristic of the leaching bacteria.
- The mining environment is characterized by the presence of high acidity and high concentrations of various metals. Hence, resistance of heavy metals and acidity are desirable traits for these bacteria to survive in the mining environments.
- In the present work two biomining bacteria isolated from different mining ecosystems were compared for their resistance to various heavy metals and arsenic in particular, in order to assess their suitability for use in mining.

AIM AND OBJECTIVE

- To compare the growth and activity of selected isolates in presence of various heavy metals.
- To study the inhibition of growth of isolates at various concentrations of arsenate and arsenite in particular.

EFFECT OF ARSENATE (As^{+5}) ON ACTIVITY OF SRD 5.

(A) 20-100 mM As^{+5}

(B) 100-1000 mM As^{+5}

EFFECT OF METALS ON HGM 26.

COPPER, ARSENIC AND NICKEL

EFFECT OF METALS ON GROWTH OF HGM 26

1. COPPER, ARSENIC AND NICKEL

2. ZINC AND CADMIUM

EFFECT OF ARSENITE (As^{+3}) ON ACTIVITY OF SRD 5.

(A) 20-100 mM As^{+3}

(B) 100-1000 mM As^{+3}

EFFECT OF METALS ON SRD 5.

COPPER, ARSENIC AND ZINC

EFFECT OF METAL ON GROWTH OF SRD 5

1. COPPER, ARSENIC AND NICKEL

2. NICKEL AND CADMIUM

EFFECT OF ARSENATE (As^{+5}) ON ACTIVITY OF HGM 26.

(A) 10-100 mM As^{+5}

(B) 100-1000 mM As^{+5}

MATERIALS AND METHODS

1. ORGANISM :
Two acidophilic, autotrophic, iron oxidizing isolates were studied. Among them SRD 5 was from polymetallic concentrate of Ambamata mine, Gujarat and HGM 26 was from Hutti gold mine, Karnataka.

2. MEDIUM:
Stock cultures maintenance and all the experiments were done in mineral salt medium (9K) given by Silverman and Lundgren (1959) at pH 1.9.

3. HEAVY METAL RESISTANCE STUDY:
Resistance to various heavy metals was studied in 250 ml Erlenmeyer flask containing 100 ml 9K medium amended with highest reported concentration of Copper, Zinc, Nickel and Cadmium Sulphate.

4. ARSENIC RESISTANCE STUDY:
Arsenic resistance was studied with Both forms of arsenic: Highly toxic Arsenite (As^{+3}) and mildly toxic Arsenate (As^{+5}).

5. ESTIMATION OF GROWTH:
Bacterial growth was monitored by counting the number of viable cells from supernatent under a light microscope using Petroff Housser counter.

6. FERROUS ESTIMATION:
Amount of ferrous in liquid media was monitored at regular intervals by titrimetric method using 0.01 N Potassium dichromate in presence of Diphenylamine indicator.

CONCLUSION

- HGM 26 showed good IOR (iron oxidation rate) in presence of 6.3 and 3 g/L arsenate (As^{+5})which was 0.718 and 1.3 g/L/d respectively,While control flask gave 2.607 g/L/d IOR.
- HGM 26 and SRD 5 showed resistance to 55 g/L and 51 g/L copper sulphate at similar scale and IOR was in range of 0.146 to 0.18 g/L/d.
- Along with iron oxidation, HGM 26 also showed 6 and 3.57 fold increase in cell load in presence of (As^{+5}), while control showed 9.57 fold increase in 6 days.
- Rest all other metals viz. Nickel, Zinc, Copper and Cadmium ad adverse effect on cell growth and iron oxidation.
- Both concentrations of (As^{+3}) severely affected iron oxidation in culture 5, where only 0.1 and 0.12 g/L/day IOR was observed.
- Cell growth in rage of 3.55 fold was observed in 6.3 g/L (As^{+5}), while a marginal 1.5 fold increase in growth was observed in presence of 51 g/L copper and 59 g/L Nickel.

ACKNOWLEDGEMENT

We are thankful to our research colleagues and teaching and non-teaching staff of the department for extending their support throughout the work.

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6-8 DECEMBER 2009

50th ANNUAL CONFERENCE OF ASSOCIATION OF MICROBIOLOGISTS OF INDIA.

National Chemical Laboratory, Pune. December 15-18, 2009

SUBSTRATE DIVERSITY AMONG IRON OXIDISERS ISOLATED FROM INDIAN MINES.

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INTRODUCTION

- There is a continuous shortfall of high-grade economic minerals for metal extraction and this is more intensely felt in developing country proceeding toward industrialisation such as India.
- As against this, many developing nations are richly endowed with plenty of lean grade mineral reserves.
- Hence, a new branch of metallurgy known as Biohydrometallurgy has developed extensively in last four decades, which has made it possible to extract metals from lean grade ores containing low amount of metals and its translation into field scale practice is easier.
- In this method, the medium of water allows the microorganisms to grow in and around metal sulfide particles and carry out extraction of metals by breaking open the sulfide matrix.
- A wide variety of microorganisms are associated in biohydrometallurgy. It is widely accepted that the complex microbial populations associated with sulfide mineral degradation in the field are dominated by microorganisms, which have sulfur and iron oxidizing capabilities.
- These bacteria are highly diverse in terms of their physiologies and phylogenetic relationships and hence in the work presented here, efforts were made to understand the diversity among bioleaching organisms isolated from mines across India, to utilize various energy sources and metal sulfides.

AIMS AND OBJECTIVES

1. To compare the substrate utilization capacity of various isolates on different energy sources.
2. To study the effect of various media ingredients on activity of the isolates.
3. To explore the ability of certain metal sulfides to work as sole energy source for leaching organisms.

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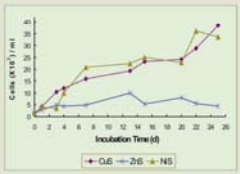

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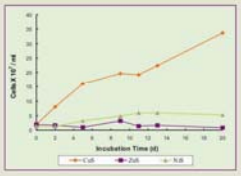

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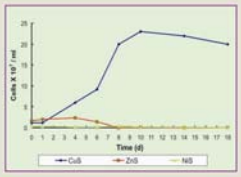

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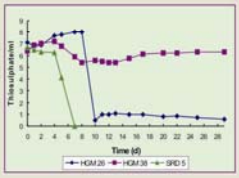

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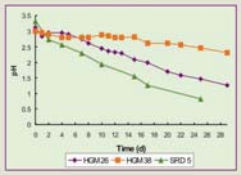


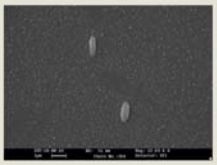
IRON OXIDATION RATE (IOR) AND SULFUR OXIDATION RATE (SOR) BY ISOLATES

No.	Isolate	Mine	State	IOR (g/l/h)	SOR (g/l/d)
1	SRD 5	Ambamata Polymetallic mine	Gujarat	0.785	3.04
2	Tf culture A			0.178	2.84
3	Tf culture E			0.228	2.47
4	HGM 17	Hutti Gold Mine	Karnataka	0.412	2.1
5	HGM 26			0.292	2.59
6	HGM 30			0.298	1.16
7	HGM 38			0.116	0.81
8	2MC	Malanjkhand copper mine	Rajasthan	0.279	0.97
9	Lig Tf	Neyveli lignite mine	Jharkhand	0.336	1.51

HGM 26 (26000 X)

HGM 38 (50000 X)

SRD 5 (22690 X)



MATERIALS AND METHODS

1. ORGANISM :
Acidophilic, autotrophic, iron oxidizing organisms isolated from water and soils samples of various mines were taken.
Three isolates were from polymetallic concentrate of Ambamata mine, Gujarat, four isolates from Hutti gold mine , Karnataka and one isolate each were taken from Malanjkhand copper mine, Rajasthan and Neyveli lignite mine, Jharkhand.

2. MEDIUM:
Stock cultures maintenance and all the experiments were done in mineral salt medium (9K) given by Silverman and Lundgren (1959) at pH 1.9.

3. ENERGY SOURCE STUDY:
Substrate diversity of the isolate was studied by adding 1% w/v concentration of thiosulfate, sulfur, copper sulfide, zinc sulfide and nickel sulfide individually in ferrous sulfate free medium.

4. SULFUR OXIDATION RATE:
Sulfer oxidation was measures by analyzing sulfate by $BaCl_2$ method.

5. ESTIMATION OF GROWTH:
Bacterial growth was monitored by counting the number of viable cells from supernatent under a light microscope using Petroff Hausser counter.

6. FERROUS ESTIMATION:
Amount of ferrous in liquid media was monitored at regular intervals by titrimetric method using 0.01 N Potassium dichromate in presence of Diphenylamine indicator.

CONCLUSION

1. Among 9 iron oxidizing isolates, highest IOR (0.785 g/L/h) and SOR (3.04) was achieved with isolate SRD 5 isolated from Ambamata mine, Gujarat.
2. Isolate SRD 5 showed a lag phase of 4 days after which it oxidised 1% thiosulfate rapidly, while HGM 26 showed a lag phase of 8 days and completed the oxidation in 10 days.
3. Isolate HGM 38 could not utilize thiosulfate at all.
4. None of the isolate tested utilized 6% thiosulphate as energy source, though isolate HGM26 and SRD 5 showed >99% oxidation of 1% Thiosulfate.
5. SRD 5 showed the rapid decline in pH which reached 0.8 in25 days, when sulfur was used as energy source. This was followed by HGM 26 which reached 1.5 pH in 29 days. In case of HGM 38, the growth as well as pH decrease was very less.
6. Isolate SRD 5 could use both copper and nickel sulfides as sole source of energy, while HGM 26 and HGM 38 could use only copper sulfide as energy source.
8. Activity of HGM 26 and HGM 38 was not much affected in presence of 4% PO_4 in medium. However isolate SRD 5 showed decreased activity in presence of 4% phosphate.
9. Addition of 5% NaCl in medium showed adverse effect on activity of all the isolates However isolate SRD 5 showed adaptation to high saline environment and better iron oxidation was seen after a lag period

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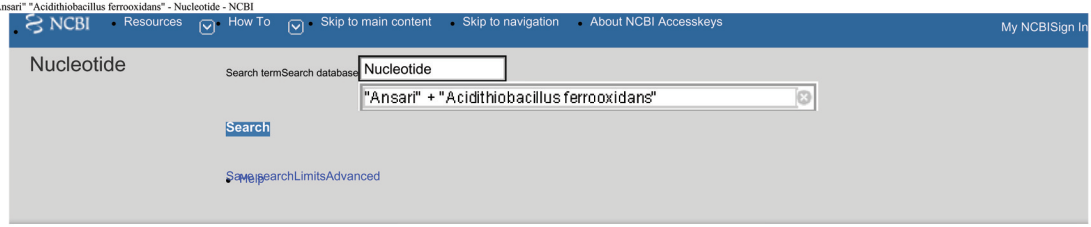
15 - 18 December 2009



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3. **Acidithiobacillus ferrooxidans** strain SRDPA3 16S ribosomal RNA gene, partial sequence
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4. **Acidithiobacillus ferrooxidans** strain SRDPA2 16S ribosomal RNA gene, partial sequence
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5. **Acidithiobacillus ferrooxidans** strain SRDPA1 16S ribosomal RNA gene, partial sequence
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GenBank: HQ262544.1

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AUTHORS     Dave,S.R. and Ansari,P.A.
TITLE       Direct Submission
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GenBank: HQ262545.1

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TITLE       Direct Submission
JOURNAL     Submitted (14-SEP-2010) Department of Microbiology, Gujarat
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Acidithiobacillus ferrooxidans strain SRDPA3 16S ribosomal RNA gene, partial sequence

GenBank: JF701443.1

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GenBank: HM116944.1

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GenBank: JF701445.1

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1   gacccttgcg taggaatctg tcttttagtg ggggacaacc cagggaaact tgggctaata
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481 ggtctgtcgt gaaatccccg ggctcaacct gggaatggcg gtggaaaccg gtgtactaga
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661 gagcaaacag gagtagatgc
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http://www.ncbi.nlm.nih.gov/nuccore/JF701445.1

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